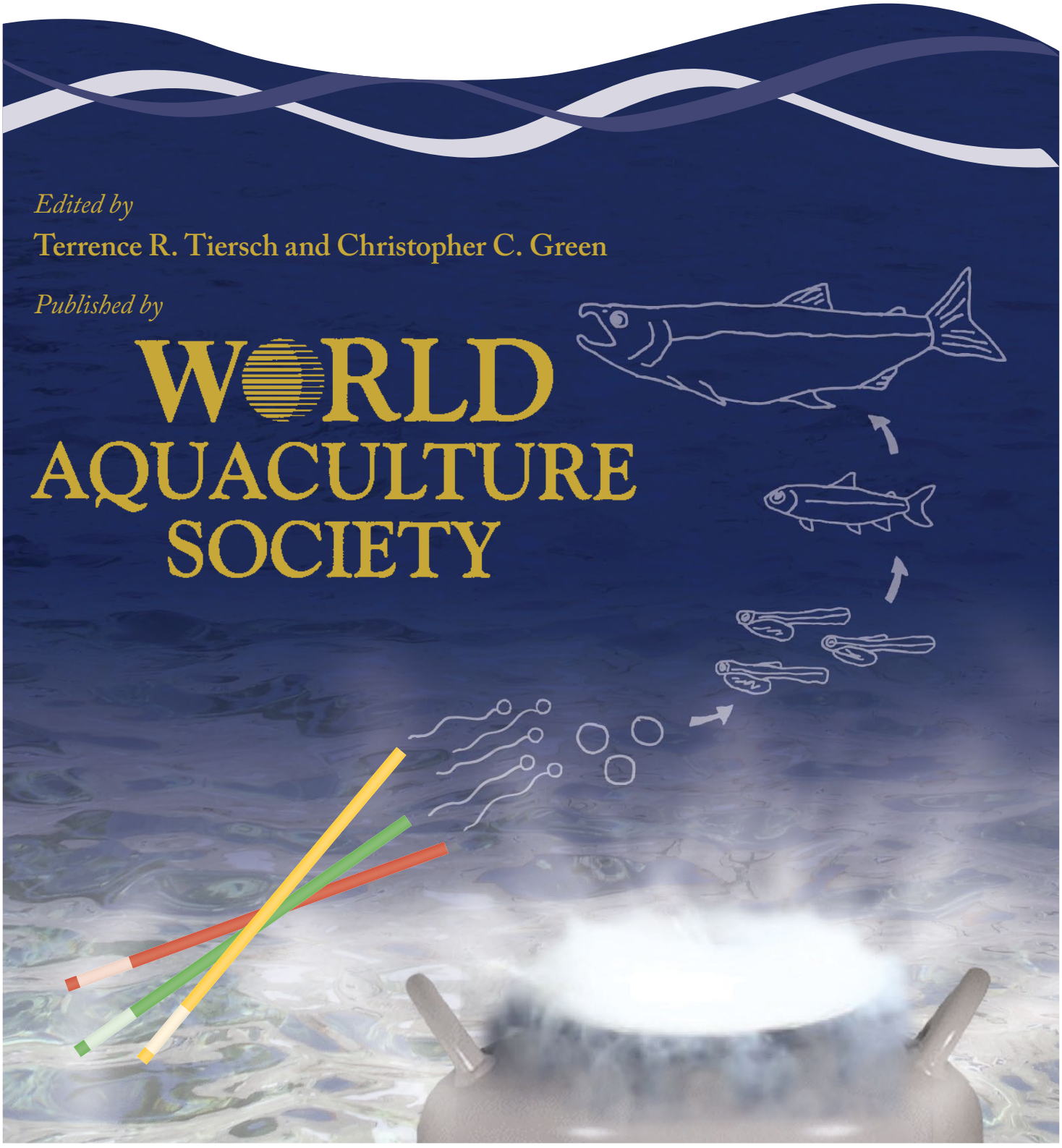
Cryopreservation in Aquatic Species

2nd Edition

A comprehensive overview of current practices, programmatic development and future directions for cryopreservation of gametes, embryos and larvae of aquatic species



Advances in World Aquaculture, Volume 7

Cryopreservation in Aquatic Species, 2nd Edition

Edited by

Terrence R. Tiersch and Christopher C. Green

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Preface to the Second Edition

Terrence R. Tiersch

The fate of this planet is governed by the demands and activities of its human population, and from these demands and activities the twin themes of the present volume emerge. We have sought to address the agricultural and conservation aspects of cryopreservation in aquatic species -- fields that are still very much in development. This book is designed as a comprehensive single-volume compendium of information on cryopreservation in aquatic species. It may be used as a textbook, general reference, or research guide. Our goal was to provide a broad overview of the principles, procedures, and perspectives necessary for development and application of the technology of cryopreservation. We have chosen to follow the guiding principle of the first edition to present not only what we know now, but also what we do not know. Thus this edition is not intended to be a final or complete work, and indeed future volumes could follow with more detailed information on genetic improvement for aquaculture and protection of natural resources, and greater emphasis on programmatic development and commercialization.

In preparing this volume, we wished to facilitate interaction among the groups needed for beneficial and effective use of cryopreservation in aquatic species. We hope that it will act as a catalyst for continued growth of an international cryopreservation community in aquatic species, and that it will serve a useful role in education and research, and most importantly, in the development of lines of communication among the different groups working in this field. These were the hopes for the first edition (its success in meeting these aspirations can be assessed by other) and remain as our primary goals for this edition.

Overview of the Contents of the Second Edition

This edition now encompasses 101 chapters (there were 55 in the first edition) organized into 11 sections (9 previously). The initial section provides basic principles for the collection and maintenance of high-quality gametes. The second section is new and addresses methods for assessment of gamete quality including new technologies in microfluidics. This area was expanded into a new section because of its importance to future development in the field. The third section addresses some basic applied aspects of cryobiology including vitrification. The fourth and fifth sections address cryopreservation of gametes and early life stages of vertebrates (expanded to cover sharks and amphibians), and the sixth Section addresses these topics including oocytes in aquatic invertebrates. The seventh section provides technical information on the full spectrum of research activities related to cryopreservation and incorporates a discussion of pathway development rather than protocol enhancements. Section eight addresses the linkage of cryopreservation to genetics and includes contributions on sample pooling, strain recovery with all-paternal inheritance, intracytoplasmic sperm injection into eggs, and the supporting role that cryopreservation can provide for other research.

Because cryopreservation is in effect a means of time travel it is necessary to be concerned with the future as well as the present. Although many aquatic species are in rapid decline, we need to develop comprehensive programs, not just emergency plans that provide temporary solutions. Section nine is in recognition that future investment of money, time, and

often priceless resources require us to ensure collection of appropriate biological data and development of effective databases. To provide specific examples, currently active germplasm repositories are described, and the regulatory aspects of sample transfers and disease prevention are discussed. This section has been expanded to include contributions on programmatic development from the public and private sectors, and from several resource centers involved in biomedical research. Section ten is new and provides updates from around the world to illustrate the needs, and type of work and programs that are being pursued internationally. Section eleven includes contributions addressing economics, cryopreservation in livestock, risk perception, conservation policy, access and benefit sharing, and high-throughput processing. With cryopreservation we literally project our mistakes forward in time. Therefore we considered it essential to provide technical information along with perspectives for the future, and to call attention to the need for a thoughtful evaluation of ethical questions.

It is important at this juncture to call attention to the fact that essentially no publications (other than this book) address the ethical considerations specifically relevant to cryopreservation of aquatic germplasm. One possible explanation for this dearth of literature is that these discussions will develop over time as cryopreservation in aquatic species becomes more visible to the public and to those involved in reproduction of amphibians, fish, and shellfish. In addition, this would involve a considerable amount of work to cover the tremendous diversity of activities involved with aquatic species ranging from conservation, hatcheries, and fisheries management (e.g., environmental ethics), to commercial aquaculture (e.g., business ethics), to disease and pathogens (e.g., veterinary ethics), to biomedical models (e.g., research ethics).

One as yet unexplored route to a straightforward formulation of relevant approaches to aquatic species cryopreservation is to look to the world of "practical ethics" (e.g., LaFollette 2003). This is a relatively new branch of ethical science that provides tools for assembling relevant fields of ethical thinking into a focused composite addressing a specific entity. For example, this approach has been used to assemble what is now recognized as the field of "science ethics" or "scientific integrity". Bodies of relevant ethical thinking such as those dealing with conflicts of interest, the definition of authorship, identifying plagiarism, protection of intellectual property, animal care and use, proper data management, and what constitutes informed consent have been assembled into a working framework that is becoming routinely accepted by researchers around the world, and is widely available in a number of textbooks (e.g., Macrina 2005) and websites (e.g., International Council of Medical Journal Editors, *www.icmje.org).* Such an approach would be an extremely valuable first step in codifying the ethical considerations material to aquatic species cryopreservation. A good example of a starting document is a recent paper that brings together the components pertinent to "agricultural ethics" (CAST 2005). *At this point, a simple consensus concerning a list of the relevant activities and ethical disciplines in the area of aquatic species cryopreservation, and eventually the broader area of aquatic germplasm and genetic resources, would constitute valuable accomplishments.*

We hope that this volume can assist development in these and other related areas, and anticipate that this volume will be used mainly in teaching, research, and program development. For use in the classroom, it could be adapted as a textbook for a one-semester, upper-division seminar course. The organization and length of this book do not readily lend themselves to the usual 15-week semester, but material can be tailored to meet the needs of the students (Table 1, next page). Classroom discussions could be strengthened by inclusion of published articles that relate to the selected weekly topics.

Table 1. Example outline for use of this book as a text in a one-semester seminar course.

Weeks	Topic	Section
1	Class overview: Preface and Introduction	I
2	Basics of aquatic species reproduction	I
3, 4	Basic principless of cryopreservation and methods	III, VII
5	Gamete quality assessment	II
6	Cryopreservation of sperm of vertebrates	IV
7, 8	Cryopreservation of eggs and embryos of fishes	V
9	Cryopreservation of invertebrates	VI
10	Data collection and databases	IX
11	Biosecurity and regulatory concerns	XI
12	Economics, valuation, and market creation	XI
13	Repository development and ethical considerations	IX, XI
14	International perspectives	X
15	High-throughput processing and pathways	XI

Researchers should have the easiest time navigating through this text. Those new to the field or working with new species can begin research efforts with approaches used for related species, although it is important to learn from one's own successes and failures rather than simply repeating the work of others. Administrators, businesses, funding agencies, and politicians could make use of this volume to gain a basic understanding of the various factors that are necessary to build a cryopreservation program. As with all technologies, most of the real work takes place after researchers have demonstrated technical feasibility. Like any other tool, cryopreservation presents costs and benefits, and is best employed with careful planning and integration with existing programs.

Notes on the Structure of the Second Edition

The first edition never appeared in electronic format, so all of the original chapters are retained in this edition in revised or annotated forms, and are interspersed with the 46 new chapters. We have attempted to clearly differentiate original and new material. The status of each chapter is indicated by a banner across the top of the first page which indicates if this is a new contribution, or an original chapter that was: 1) revised by the authors, 2) annotated by the original authors, or 3) annotated by the Editorial Board to encompass relevant developments in the past 10 yr. The annotated bibliographies appear under a line at the end of the original references, and include articles published since 2000 as well as other useful articles published before then that were not included in the original References. For the most part, obvious cross-references among chapters were not made in the text because electronic formatting allows easy searches within the document. It is for this reason that an Index was not included with this edition. Each chapter was intended to be self-contained and therefore includes a References section and an auto-citation statement at the bottom of the first page. However, the author addresses are located in the front of the document rather than in each chapter. There is a single video in this edition found in the last chapter (accessible by a link in the legend for Figure 1) that shows the operation of high-throughput processing equipment.

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Acknowledgements for the Second Edition

Aquatic species cryopreservation has expanded greatly since the publication of the first edition of the book in 2000 and is rounding into a field of study in its own right. The volume of the associated scientific literature has doubled in that time and a number of high-profile programs have begun including the creation of the National Animal Germplasm Program by the United States Department of Agriculture. This second edition is intended to integrate that development and to point toward the future. Production of this edition has been a large undertaking. The new material in this edition is by itself larger than the original book, and the original chapters have all been revised or annotated. Given the size of this volume and our desire to make the information widely available, we decided to prepare it as an electronic (CD) version only.

Beyond the challenges posed by the scope of this edition, this project was delayed by the effects of two destructive hurricanes here in Louisiana, and represents 2.5 yr of work by a very dedicated group of people. First we thank the 97 authors involved in the writing of new chapters or revising of original chapters. They were a pleasure to work with. Our Editorial Board included Huiping Yang, Jon Daly, Mike Christensen, Rafael Cuevas-Uribe, Noel Novelo, E Hu, Doug Kuenz, and Shaunna Harris. We are very grateful for their many hours of volunteer service to this project. Others making valuable contributions to the production of this edition were Christian Quitadamo, Kouassi Da Costa, Katherine Gautreaux, Kenneth Whitfield, Harvey Blackburn, and John Hargreaves. We are especially grateful to Kim Daly for creating the cover art for the CD.

We are also grateful for reviews by colleagues in federal and state agencies, academic institutions, and the private sector. Craig Browdy, Book Editor for the World Aquaculture Society, assisted with production. We thank Ron Becker and Chuck Wilson of Louisiana Sea Grant for their continued support of this work. We worked very hard to try to communicate with all original authors, but this was not possible in all cases. We apologize to anyone that we missed who would have wanted to contribute to this edition. This volume will make its debut at a special session to be held March 2011 in New Orleans during the annual meeting of the United States Chapter of the World Aquaculture Society.

This second edition is dedicated to Connor and Nolan Tiersch (who are now old enough to understand this work) and to all those who will inherit the results of decisions we make as individuals or as groups, and those decisions that we do not make. The use of cryopreservation continues to present us with a challenge to link wisely the past, present, and future.

Mardi Gras 2011

Terrence R. Tiersch
Christopher C. Green

Acknowledgements for the First Edition

This volume is the result of several events. The first is the Fish Sperm Cryopreservation Conference held at Louisiana State University in the fall of 1995 with financial support from the U. S. Fish and Wildlife Service (USFWS) and assistance from Vincent Mudrak. That conference underscored the need for compilation of the various aspects of cryopreservation in aquatic species and provided the opportunity for the discussions that served as a foundation for the volume. The second is a special session, *Application of Fish Sperm Cryopreservation*, organized by TT and PM, held at the 1997 meeting of the World Aquaculture Society. Speakers focused on the transition from cryopreservation research to application, and the ensuing discussions addressed the development of this volume. A call for contributions was made, and soon after, William Knapp, Chief, Division of Hatcheries, USFWS, approved allocation of funds for publication. The third is a special session, *A Broader View of Cryopreservation*, organized by TT and PM, held at the 1998 meeting of the World Aquaculture Society. Discussion after the session led to coalescence of the ideas represented in this volume, and soon after, arrangements were finalized for publication by the World Aquaculture Society. The volume will make its debut at a special session to be held February, 2000 in New Orleans during the annual meeting of the U. S. Chapter of the World Aquaculture Society.

In addition to the 68 authors, the final product reflects the efforts of at least an equal number of people who helped with the reviewing and production. The editors thank John Hargreaves and Stephen Wachtel for their critical discussions, and James W. Avault Jr., John Chandler, Joseph Cloud, Konrad Dabrowski, Edward Donaldson, Harry Dupree, George Gee, Robert Godke, Lawrence Johnson, William Knapp, Stanley Leibo, Esther Lubzens, Vince Mudrak, Robert Romaire, Harald Rosenthal, Gary Thorgaard and William Wolters for their valuable advice. Others deserving recognition include Ron Becker (Associate Executive Director, Louisiana Sea Grant College Program), William Brown (Associate Director, Louisiana Agricultural Experiment Station), John Buchanan, Rex Caffey, Richard Denniston, Chester Figiel, Jill Jenkins, Rebecca Nestor, Carmen Paniagua, William Wayman and Gang Yu.

We are grateful for reviews by colleagues in federal and state agencies, academic institutions and the private sector. Craig Browdy, Book Editor for the World Aquaculture Society, assisted with editing and production. We are especially grateful for the devoted efforts at West Virginia University of Ronnie L. Dumire Jr., computer specialist, and Rebecca D. M. Smith, graduate student, who prepared the text.

This volume is dedicated to Connor and Nolan Tiersch and to all those who will inherit the results of our decisions. The use of cryopreservation presents us with a challenge to link wisely the past, present and future.

December 1999

Terrence R. Tiersch
Patricia M. Mazik

Foreword to the First Edition

William E. Knapp
(Former Chief, Division of Fish Hatcheries, U.S. Fish and Wildlife Service)

Most of us have seen those television commercials where bustling people suddenly become motionless and silent upon someone's mention of "E. F. Hutton", an investment firm whose financial advice is presumably so highly regarded as to freeze even the busiest among us. But most of us are probably unaware that the "E. F. Hutton" of the Pacific Northwest has recent spoken clearly about the valuable roles that cryopreservation and experimentation must play in the future of hatchery operations.

The Northwest Power Planning Council recently reported to the United States Senate that "The stereotype of the hatchery and production manager pumping out fish for possible harvest opportunities without awareness of the environmental context of that production or concern for the potential ecological effects no longer exists." The Council went on to note the valuable roles that cryopreservation techniques and hatchery experimentation play in preserving species faced with imminent demise and stressed that without immediate protection, those species will become extinct.

Many of us in the fish business have been delivering these messages for the better part of the last decade, but have not succeeded in freezing resource managers and politicians long enough to get their attention. Perhaps now that an organization of stature has delivered those same messages to the Congress of the United States, the role of cryopreservation as a tool in managing wild and hatchery fish will be better understood and appreciated, and accepted more broadly.

When you cut to the chase, cryopreservation of the reproductive products of fish is essential for four separate but related reasons. First, as the number of species listed as threatened or endangered grows and, more importantly, as prospects for recovering those species in the near-term diminish, it is increasingly important that fishery biologists and fishery managers take advantage of all of the tools at their disposal to conserve species and biological diversity. In this regard, while cryopreservation remains an imperfect tool in some respects, it nonetheless offers unique opportunities to preserve genetic material and thus conserve genetic diversity. Second, as requirements for maintaining captive broodstock and for selecting brood fish from the wild become increasingly stringent, with respect to ensuring genetically representative parents and avoiding adverse effects on wild fish, it is desirable to have genetic "reserves" on hand cryopreserved. Those reserves can be invaluable in optimizing the genetic diversity of young fish produced by following carefully developed mating strategies, thus enhancing their fitness and prospects for survival in the wild. Third, as requirements for maintaining captive broodstock become more complex and necessitate having more space available at hatcheries to maintain distinct broodstock in isolation, as is happening for Atlantic salmon in hatcheries in Maine and for Pacific salmon throughout the Northwest, it becomes increasingly advantageous to have genetic reserves on hand to reduce needs to maintain live brood fish on station. Fourth, and perhaps most important, as both our knowledge of fish genetics and our capabilities to genetically engineer fish expand, it simply makes good sense to have available repositories of genetic material that are as diverse as possible, especially since diversity among and within species in the wild continues to decline.

So why has interest in applying the principles and techniques of cryopreservation not met with the same warm reception that greeted earlier efforts to apply this technology to plants, livestock, and mammals and birds of ecological importance? And why are seed banks so common in agriculture, horticulture and other botanical areas and in management of captive animals in zoos, but not in fishery management in general and captive propagation of fish in particular? While there are several explanations, the one that most often limits the application of cryopreservation in fishery management is the fear that use of this tool to provide a genetic "safety net" will somehow compromise ongoing efforts to conserve and restore habitat. This concern can be traced back to the belief that it was the availability of captive propagation that encouraged wanton and reckless destruction of native habitats and over-harvest of wild fish throughout this country for the past 130 yr. When this belief is examined closely, two things become clear. First, our forefathers' faith in the abilities of hatcheries to produce large numbers of fish did, in fact, contribute to the widely held notion that hatcheries could compensate for severe reductions in natural populations. Second, even if that faith had not burned warmly, our ancestors would have nonetheless harnessed waterways for commerce, flood control, navigation and power. Their unbridled and relentless desires to manifest their destiny and exercise dominion over nature would have obscured and overshadowed their concerns about maintaining viable fisheries, and, in fact, did in many places regardless of their often misplaced faith in hatcheries.

Today we know there are no "free lunches" when it comes to managing and appropriating water resources. While captive propagation and cryopreservation serve as valuable tools for conserving and maintaining biological diversity, there is little argument in the fishery science and fishery management communities that the tool of first choice is protecting natural habitat and, wherever possible, restoring native habitats. Nonetheless, opposition to developing and applying cryopreservation remains, predicated on the belief that having a versatile tool of this type encourages decision-makers to disregard the importance of conserving and restoring habitat. This is not so.

The voice of reason is being heard more loudly and clearly, and the right messages are getting to the right people with increasing frequency. Cryopreservation works and more experimentation is needed to perfect this tool. Cryopreservation does not threaten nor conflict with habitat protection and restoration; rather, it complements it. At the same time, cryopreservation is not a universal cure for all that ails our nation's fisheries. In the hands of the right professionals and used in the right ways, cryopreservation can work to conserve genetic diversity and enhance the fitness and survival of wild and hatchery fish.

It is for this reason that the U.S. Fish and Wildlife Service, Division of Fish Hatcheries, provided funding for this first volume on cryopreservation in aquatic species. It is our hope in compiling and making this information available that advances can be realized and applied to the safeguarding of our fishery resources for the future.

Introduction to the Second Edition

Terrence R. Tiersch

Aquatic species cryopreservation has grown substantially in the past decade since publication of the first edition of this book. Cryopreservation has progressed from its original status as something of a research diversion or oddity, to a quirky unexploited technology with unrealized promise, to its current status as a viable strategy available for incorporation into broader programmatic frameworks as a supporting technology, with the potential to launch commercial development into new areas such as improvement, maintenance, and distribution of genetic resources. Despite this vigorous growth and progress, problems still remain before repositories based on cryopreservation can begin to provide more than basic utility, typically in self-contained programs. Successful entities and activities need to develop interconnections; projects must combine into programs which can coalesce into systems capable of crossing from research to large-scale application and cooperation across commodities and countries. As such, a roadmap is needed to identify routes available for future application of this technology.

In addition, scientific study in this area, although burgeoning, requires a blueprint to lay out plans for unification and codification as a true research field. "Those attempting to utilize the available literature on cryopreservation in aquatic species will encounter a number of problems. The literature is distributed across numerous journals and disciplines, and there is a lack of standardization in terms, protocols and reporting of results. Those new to the field are often confronted with successful protocols that cannot be repeated, unsuccessful experiments that cannot be interpreted, and contradictory findings even within a single species." Such was the assessment of the state of the scientific literature for aquatic species cryopreservation in the first edition of this volume in 2000. With the rapid expansion of this literature in the past decade (see below), this situation could be considered to be even worse today.

Moreover, cryobiology remains a developing science with a rudimentary theoretical framework, and cryopreservation research is often empirical with advances made by trial and error. It should also be noted that the term "fish" is an artificial collective of more than 25,000 species characterized more by differences than by similarities. To discuss cryopreservation within fish or aquatic species is thus a balancing act of attempting to generalize observations into basic principles while recognizing the considerable diversity that exists across these organisms. As such, a basic philosophical approach should be developed by those working in the field. Until a consensus can be reached, an initial working approach could include the following concepts:

1) Be aware of the differences among entities such as species and user groups;
2) Focus on the commonalities across groups and technologies;
3) Generalize technology development to the extent possible;
4) Target broad application of findings, and
5) Work to reduce barriers to communication and integration across communities (e.g., species, commodity groups, or private and public sectors).

Further compounding these difficulties in utilizing the aquatic species cryopreservation literature is the lack of standardization in protocols, terminology, and reporting. Because of this hodgepodge, it is problematic or impossible to make valid direct comparisons among the results of most published studies (Table 1).

Tiersch, T. R. 2011. Introduction to the Second Edition. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 1-17.

Table 1. Examples of major factors under-appreciated for aquatic species that need to be defined, controlled, and reported to enable direct comparisons of sperm cryopreservation results for standardization and application (based on Yang et al. 2010).

Step or process	Factors to be defined	Relevance to cryopreservation
Source of animals, housing, and conditioning	Strain and source	Variation among populations or mutant lines can influence results
	Size and age	Can affect gamete quality and quantity (report body weight, length, age)
	Maturity	Reproductive condition (report sperm volume, or testis mass, and GSI*)
	Culture conditions	Water quality parameters, temperature, salinity, and light:dark cycles
	Male selection	Using best males may not represent normal variation (report selection criteria)
Sample handling, preparation, and freezing process	Initial quality	Major influence on post-thaw quality (requires assessment and definition)
	Sperm density	Can affect cryopreservation and fertilization (a major uncontrolled variable unless set)
	Cryoprotectant	Type and final concentration is a critical factor (should be defined and reported)
	Motility	Sperm motility prior to addition of cryoprotectant (should be defined and reported)
	Equilibration time	Requires tight control of methods and temperature (should be defined and reported)
	Packaging	Affects heat transfer (type, size, and materials should be defined and reported)
	Biosecurity	Such as sealing of containers and disinfection (should be defined and reported)
	Cooling rate	Critical cryobiological factor (should be defined with start and finish temperatures)
	Storage time	Duration can differentiate freezing and super-cooling (should be defined and reported)
Egg collection and use of thawed sperm samples for fertilization	Pooling of eggs	Sometimes used to provide sufficient numbers for experiments (should be reported)
	Thawing process	Warming temperature, duration, and rate (should be defined and reported)
	Post-thaw motility	Necessary to estimate effects of cryopreservation (should be defined and reported)
	Fertilization method	Can influence gamete activation especially for thawed sperm (should be reported)
	Sperm-to-egg ratio	Concentration and volume of sperm for fertilization (should be defined and reported)
	Egg quality	Fresh sperm can be used to evaluate fertility of eggs (should be reported)
	Fertilization rate	Reporting of exact definition of fertility criteria should be compulsory
	Hatching rate	Reporting of absolute or relative values should be identified (report both)

*GSI, gonadosomatic index, the percentage of testis weight in relation to the body weight.

More insidious is the problem caused by usage of particular terms such as “percent motility” or “percent fertilization” to represent a spectrum of widely varied activities and endpoints that are partially reported or not defined; such terms are often directly compared with the assumption that the conclusions are meaningful. This is a basic impediment to the pursuit of scientific research and has been addressed in a number of sections and chapters throughout this volume.

In brief, cryopreservation addresses the freezing, cryogenic storage and thawing of living material. Gametes or early life stages (e.g., embryos and larvae) are collected and suspended in an extender solution. The material to be frozen is usually evaluated for quality (e.g., motility of sperm) and can be maintained by refrigeration prior to the actual freezing and thawing processes. Fertilization success of gametes and subsequent development of early life stages are the first demonstration of cryopreservation success. Numerous factors such as cooling and thawing rates can influence formation of ice crystals, cell dehydration and maintenance of cell integrity. The addition of permeating cryoprotectant agents (e.g., dimethyl sulfoxide, methanol or glycerol) can minimize cell damage associated with ice formation. Most cryoprotectants, however, are toxic to cells and must be diluted with an extender solution prior to the addition of sperm. Cryoprotectant concentration and equilibration time (for the cryoprotectant to permeate the cell) can influence cryopreservation success. Moreover, this process can be species-specific or male-specific (although the exact magnitude and sources of variation are yet to be identified), and it can even depend on the timing when sperm are collected during the spawning season. These factors can lead to fertilization success rates that are variable among and within species. Previous reviews on fish sperm cryopreservation notwithstanding (e.g., Horton and Ott 1976, Scott and Baynes 1980, Stoss 1983, Billard et al. 1995, Rana 1995, Tiersch and Mazik 2000, Cabrita et al. 2009) the literature on this subject remains fragmented and disjointed, and protocols for cryopreservation vary considerably from study to study.

With respect to the human dimension, cryopreservation researchers working with aquatic species comprise a heterogeneous lot. Consider the variation presented by the authors of this volume, who likely provide a representative sample (perhaps even a healthy percentage) of the total population of active workers around the world. The more than 140 authors represent more than 20 countries (including some 25 states in the United States) and at least 75 different organizations and departments ranging from veterinary and medical schools to land-grant schools and community colleges to tribal governments to state, provincial and federal agencies to private organizations. Numerous disciplines are represented including conservation and molecular genetics, animal breeding, histology, endocrinology, physiology, basic cryobiology, veterinary medicine, animal science, oceanography, fisheries, hatcheries and aquaculture. These authors report on more than 70 species of amphibians, fishes, bivalves, gastropods, crustaceans and polychaetes. In contrast, much larger, more homogeneous groups often pursue study of cryopreservation within a single species (e.g., cattle, swine, or humans).

These and other issues have driven the genesis of this second edition. This volume recognizes protocol development as the foundation for the current status of this field, but places it in the context of pathway development (rather than the typical, narrow, single-entity research approach), and proceeds from there into commercial application and programmatic development. These activities will require a broad, comprehensive picture to emerge as this field moves forward, and it is hoped that this volume can at least serve to open some of the necessary discussions. This edition has added two new sections (gamete quality analysis, and international perspectives) and is not directly organized along the steps in the cryopreservation process. As such, cryopreservation process steps are presented across multiple sections (Figure 1).

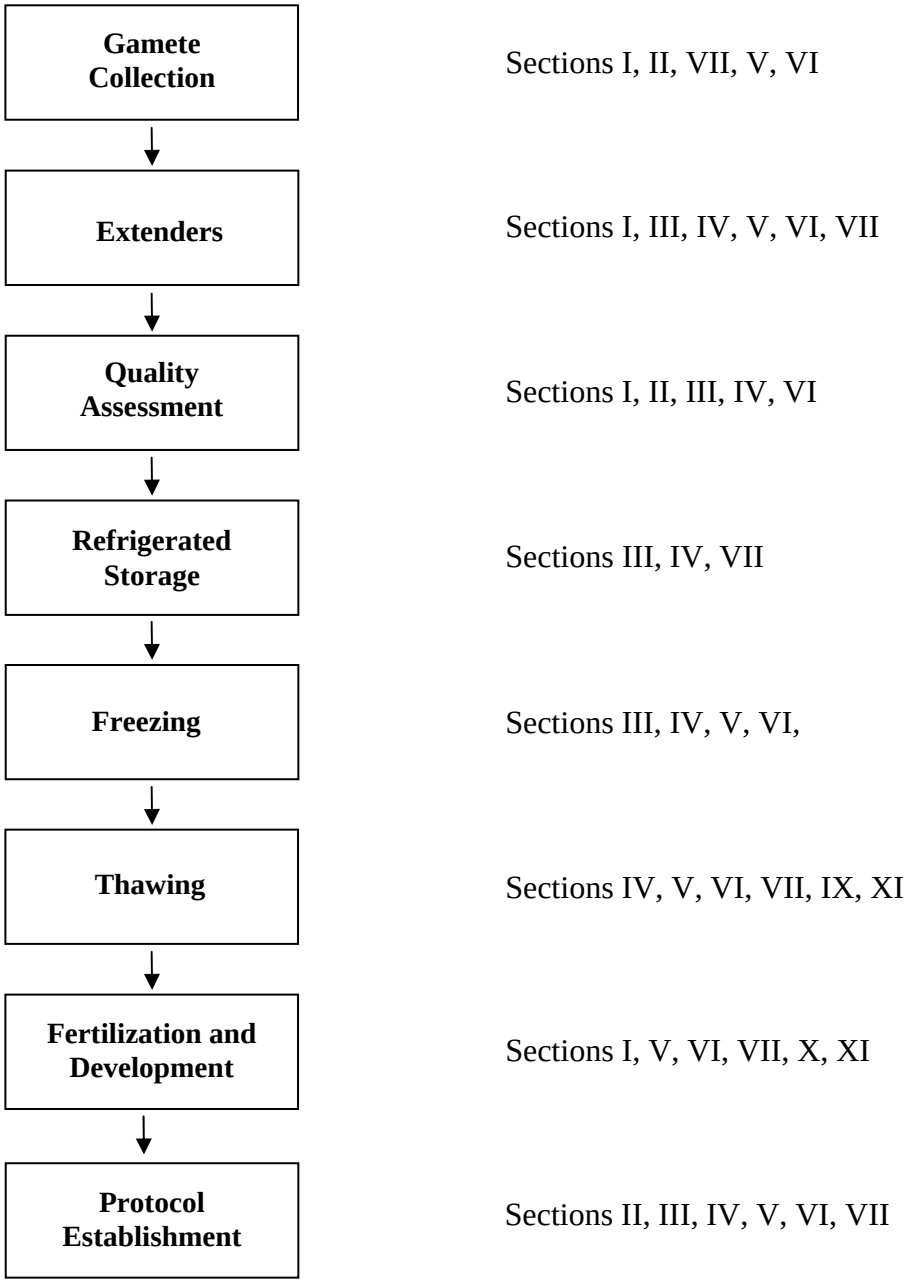

Figure 1. An outline of major steps in cryopreservation process and the corresponding sections in this volume in which the material is presented.

Likewise, this volume addresses multiple steps required for technology application and industry and programmatic development. This information is also distributed across the various sections of the volume (Figure 2, next page).

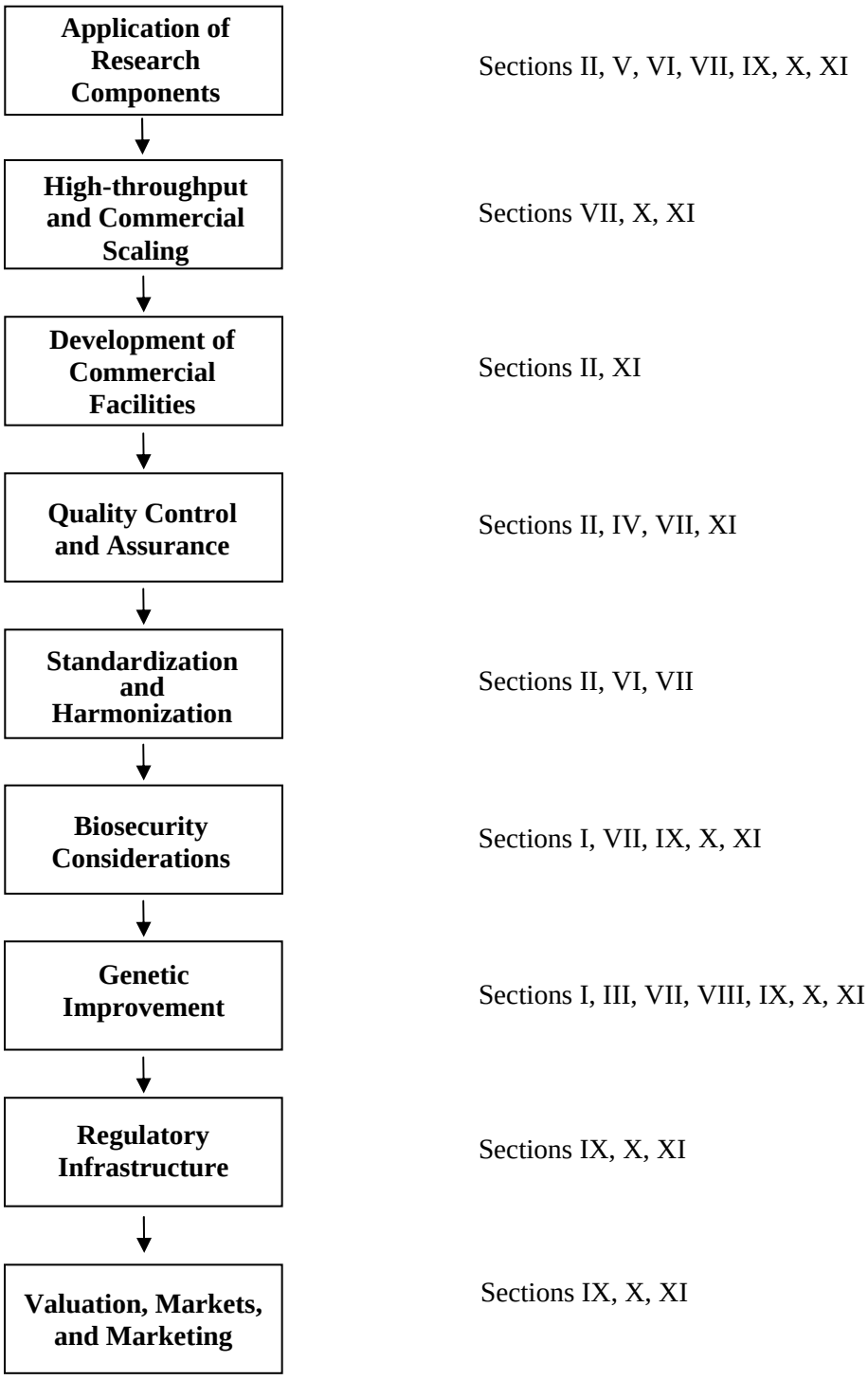

Figure 2. An outline of major steps in application and programmatic development and the corresponding sections in this volume in which the material is presented.

Despite the problems described above, cryopreservation has been successfully applied, at least in sperm, in numerous aquatic species. In many cases research groups have reinvented

protocols or developed alternative methods to yield comparable results. Fortunately, to facilitate the transition in aquatic species to commercial-scale application there are multi-million dollar industries already in place for cryopreservation of livestock semen which can provide methodologies, equipment, and insights. Cryopreservation of fish gametes gradually increased in the four decades since Blaxter (1953) reported the freezing of sperm to hybridize spring and fall spawning herring. Within the past 15 yr, the number of studies has expanded to the point where considerable uncertainty exists even in the number of aquatic species for which sperm has been cryopreserved (reported for example as between 50 and 200 species) and a current estimate is needed. Cryopreservation technology has enhanced hatchery and aquaculture operations by providing flexibility in spawning of females, greater control in breeding programs, and the ability to store favorable genes for extended periods. In addition, concern for native fish populations has resulted in examining sperm cryopreservation as a way to preserve genetic material and transfer genes between wild and hatchery populations.

A review on cryopreservation of fish sperm in the Introduction of the first edition of this book summarized 185 reports (including abstracts, conference proceedings, technical reports, book chapters, and 138 peer-reviewed journal articles) published between 1953 and 1996. It was found that research on sperm cryopreservation had at that point been described in print for at least 83 fish species from 35 families. The majority of publications were on economically important species, focused primarily on the salmonids, cyprinids and catfishes. These studies addressed freshwater (49%), marine (31%), brackish (7%) and anadromous (13%) fishes, or when viewed by categories, commercial and sport fisheries (51%), cultured ornamental and food fishes (39%), wild (non-sport) fishes (7%) and threatened and endangered species (3%). These basic trends likely hold true today, but there has been a great increase in the global nature of this work and expansion into amphibians and invertebrates. It would be very useful for cross-sector stakeholders to gather and census the numbers, types, and activity levels of global efforts in aquatic species. *Indeed, it is likely that an international society and journal could now be developed in the area of aquatic germplasm and genetics.* A basic illustration of this growth can be seen in the number of peer-reviewed publications addressing cryopreservation, which since the year 2000 is equal or larger to the number of all publications prior to that time (Figure 3).

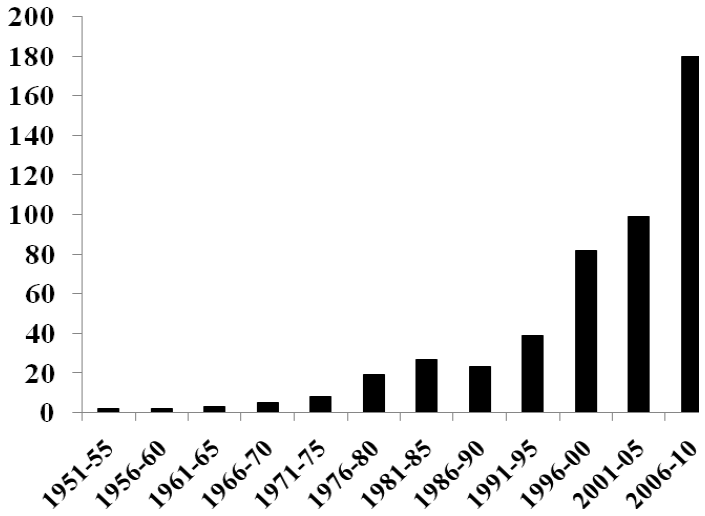

Figure 3. Six decades of peer-reviewed publications addressing fish sperm cryopreservation.

As the gaps in communication within the international community become ever smaller, increased exchange of information will accelerate research and application in cryopreservation. We should be aware that application will bring the potential for rapid changes in other fields. For example, cryopreservation can assist development and distribution of improved lines, including those produced by gene transfer. Therefore the availability of cryopreservation could accelerate application and distribution of genetically modified organisms. It is not unreasonable to assume that new product forms will emerge and current regulations may not be adequate. For example, consider the ease of transport of millions of transgenic oyster embryos in a few frozen straws compared to the transport of even a few hundred broodstock oysters. We have also seen a great increase in interest from members of the biomedical fish community since 2000. Large genetic screening projects utilize mutagenesis strategies that can yield thousands of new research lines, and this coupled with transgenesis and other technologies has produced a conservatively estimated current inventory of some 20,000 research lines maintained as live populations in zebrafish *Danio rerio* alone.

Given that cryopreservation is not perfected as yet, we should give consideration to the use of low-quality samples including non-motile sperm. Techniques such as intracytoplasmic injection (ICSI) of sperm can allow fertilization that would otherwise not be possible. There is, moreover, reason to suggest (while it is perhaps surprising to do so in a book on cryopreservation) that most germplasm repositories based on cryogenic storage in liquid nitrogen will eventually fail. Technical problems, accidents, loss of key personnel, political pressures, and changes in priorities can result in loss of cryogenic repositories. These problems would be compounded in developing countries where the expense of liquid nitrogen could inhibit repository maintenance. Efforts should be made to identify methods that complement cryogenic storage. Development of techniques such as ICSI for use in aquatic species could thus not only reclaim damaged sperm, but would also open the door to use of other less-costly methods of storage such as freeze-drying.

Fortunately, as indicated above, the published resources in aquatic species and for cryopreservation in general have expanded greatly since 2000. There are currently at least 30 high-quality reference works available in book form (most appearing in the past 10 yr) that address cryobiology, cryopreservation technology, and spermatology (Table 2, next page). In addition, there are now at least 60 reviews in a variety of forms specifically addressing relevant topics in aquatic species (Table 3). This provides a wealth of information for students and practitioners.

The benefits of cryopreservation as they are typically currently viewed include at least five aspects of improvement for existing industries (some indicated above) and for creation of new industries. First, cryopreservation can be used to improve existing hatchery operations by providing sperm on demand and simplifying the timing of induced spawning. Second, frozen sperm can enhance efficient use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males, potentially freeing resources for use with females and larvae. Third, valuable genetic lineages such as endangered species, research models, or improved farmed strains can be protected by storage of frozen sperm. Fourth, cryopreservation opens the door for rapid genetic improvement. Frozen sperm can be used in breeding programs to create improved lines and shape the genetic resources available for aquaculture. Finally, cryopreserved sperm of aquatic species will at some point become an entirely new industry itself.

Table 2. Books addressing topics relevant to cryobiology and cryopreservation including those addressing aquatic species.

Title	Citation
Life and Death at Low Temperatures	Luyet and Gehenio 1940
Biological Effects of Freezing and Supercooling	Smith 1961
Cryobiology	Meryman 1966
Current Trends in Cryobiology	Smith 1970
The Frozen Cell	Wolstenholme and O'Connor 1970
The Freezing of Mammalian Embryos	Elliott and Whelan 1977
Low Temperature Preservation in Medicine and Biology	Ashwood-Smith and Farrant 1980
ATCC Preservation Methods: Freezing and Freeze-Drying	Simione and Brown 1991
Fish Evolution and Systematics: Evidence from Spermatozoa	Jamieson 1991
Advances in Low-Temperature Biology	Steponkus 1993
Cryopreservation and Freeze-Drying Protocols	Day and McLellan 1995
Reproductive Tissue Banking: Scientific Principles	Karow and Critser 1997
Action Before Extinction	Harvey et al. 1998
Cryopreservation in Aquatic Species (first edition)	Tiersch and Mazik 2000
Cryobanking the Genetic Resource: Wildlife Conservation for the Future?	Watson and Holt 2001
Low Temperature and Cryogenic Refrigeration	Kakac et al. 2003
Life in the Frozen State	Fuller et al. 2004
The Sperm Cell	De Jonge and Barratt 2006
Spermatology	Roldan and Gomendio 2007
Advances in Biopreservation	Baust and Baust 2007
Cryopreservation and Freeze-Drying Protocols, 2nd edition	Day and Stacey 2007
Vitrification in Assisted Reproduction	Tucker and Liebermann 2007
Theory and Techniques of Fish Spermatozoa and Embryos Cryopreservation	Chen 2007
The Fish Oocyte	Babin et al. 2007
Fish Spermatology	Alavi et al. 2008
The Effects of Low Temperature on Biological Systems	Grout and Morris 2009
Sperm Banking: Theory and Practice	Pacey and Tomlinson 2009
Sperm Biology	Birkhead et al. 2009
Methods in Reproductive Aquaculture Marine and Freshwater Species	Cabrita et al. 2009
Reproductive Biology and Phylogeny of Fishes, Volumes A and B	Jamieson 2009
Fundamentals of Cryobiology	Zhmakin 2009
Fertility Cryopreservation	Chian and Quinn 2010
WHO Laboratory Manual for the Examination and Processing of Human Semen	World Health Organization 2010

Table 3. Book chapters, review articles, workshop proceedings ("W Proceeding"), and conference proceedings ("C Proceeding") addressing topics relevant to cryobiology and cryopreservation of aquatic species.

Short Title	Type	Citation
Cryopreservation of fish spermatozoa and ova	Article	Horton and Ott 1976
Cryogenic preservation of fish and mammalian spermatozoa	Article	Mounib 1978
Cryopreservation of the sperm of some freshwater teleosts	Article	Stein and Bayrle 1978
Some data on gametes preservation and artificial insemination in teleost fish	C Proceeding	Billard 1978
Reproduction and artificial insemination in teleost fish	C Proceeding	Billard 1980
Preservation of gametes of freshwater fish	C Proceeding	Erdahl and Graham 1980
A review of the biology, handling and storage of salmonid spermatozoa	Article	Scott and Baynes 1980
Cryogenic storage of gametes of carps and catfishes	Article	Withler 1980
Cryopreservation of spermatozoa of freshwater fishes of Asia	Article	Withler 1981
Cryobiology and the storage of teleost gametes	C Proceeding	Harvey 1982
Cryopreservation of fish sperm	Chapter	Kopeika and Novikov 1983*
Fish gamete preservation and spermatozoan physiology	Chapter	Stoss 1983
Cryopreservation and fertility of fish, poultry and mammalian spermatozoa	C Proceeding	Graham et al. 1984
Some factors affecting the preservation of salmonid spermatozoa	Article	Erdahl et al. 1984
Artificial insemination and gamete management in fish	Article	Billard 1988
Artificial insemination and the preservation of semen	Chapter	Watson 1990
Live preservation of fish gametes	Chapter	Leung and Jamieson 1991
Fish sperm cryopreservation in Taiwan	Article	Chao 1991
Conservation and preservation of genetic variation in aquatic organisms	Chapter	McAndrew et al. 1992
Cryopreservation of aquatic gametes and embryos	C Proceeding	Rana 1995a
Cryopreservation of fish spermatozoa	Chapter	Rana 1995b
Preservation of gametes	Chapter	Rana 1995c
Cryopreservation of fish spermatozoa: effect of cooling methods	C Proceeding	Rana and Gilmour 1996
Cryopreservation of fish semen	C Proceeding	Maisse 1996
Cryopreservation of finfish and shellfish sperms	Article	Chao 1996
Cryopreservation of embryos in the oyster and clam	Article	Chao et al. 1997
Cryoconservation du sperme et des embryons de poissons	Article	Maisse et al. 1998
Cryopreservation and aquaculture: a case study with penaeid shrimp larvae	Article	Subremoniam and Arun 1999
Cryopreservation in Aquatic Species	Book	Tiersch and Mazik (editors) 2000
Cryopreservation of gametes in aquatic species	Special issue	Lahnsteiner (editor) 2000
Techniques of genetic resource banking in fish	Chapter	Billard and Zhang 2001

Short Title	Type	Citation
Cryopreservation of finfish and shellfish gametes and embryos	Article	Chao and Liao 2001
Cryopreservation in aquarium fishes	Article	Tiersch 2001
Main improvements in semen and embryo cryopreservation for fish and fowl	W Proceeding	Blesbois and Labbe 2003
Biosecurity and regulatory considerations for aquatic species	Chapter	Tiersch and Jenkins 2003
Cryopreservation of gametes and embryos of aquatic species	Chapter	Zhang 2004
Cryopreservation of semen of the *Salmonidae*	Chapter	Lahnsteiner 2004
Aspectos generales de la crioconservacion espermatica en peces teleosteos	Article	Medina-Robles et al. 2005
Extenders and cryoprotectants on fish spermatozoa cryopreservation	Article	Muchlisin 2005
Semen cryopreservation in catfish species	Article	Viveiros 2005
Cryobanking of fish somatic cells	Article	Mauger et al. 2006
Evaluation of the damage in fish spermatozoa cryopreservation	Article	Li et al. 2006
Cryopreservation of fish sperm	Chapter	Kopeika et al. 2007
Sperm cryopreservation in fish and shellfish	C Proceeding	Tiersch et al. 2007
Low-temperature preservation of fish gonad cells and oocytes	Chapter	Zhang et al. 2007
Theory and Techniques of Fish Spermatozoa and Embryos Cryopreservation	Book	Chen (editor) 2007**
On the biology of fish sperm	W Proceeding	Rosenthal (editor) 2008
Strategies for commercialization of cryopreserved fish semen	Article	Tiersch 2008
Fish sperm cryopreservation in France	C Proceeding	Haffray et al. 2008
Role of bacteria in the chilled storage and cryopreservation of sperm	Chapter	Nimrat and Vuthiphandchai 2008
Variability of sperm quality after cryopreservation in fish	Chapter	Kopeika and Kopeika 2008
Methods in Reproductive Aquaculture Marine and Freshwater Species	Book	Cabrita et al. (editors) 2009
Sperm quality and cryopreservation of Brazilian freshwater fish species	Article	Viveiros and Godinho 2009
Criopreservacion de gametos y embriones	Chapter	Herraez 2009
Prospects and development in fish sperm and embryo cryopreservation	Chapter	Robles et al. 2009a
Germplasm cryobanking in aquarium model species	Article	Robles et al. 2009b
Live preservation of fish gametes	Chapter	Gwo et al. 2009
Current status of sperm cryopreservation in biomedical research fish models	Article	Yang and Tiersch 2009
Sperm proteins in teleostean and chondrostean fishes	Article	Li et al. 2009
Criopreservacion para la conservacion y produccion de organismos marinos	W Proceeding	Proyecto FONDEF 2009
Cryopreservation of fish gametes and embryos	Article	Diwan et al. 2010
On the biology of fish gametes	W Proceeding	Rosenthal et al. (editors) 2010

* In Russian; **In Chinese

Future development of a germplasm industry will require integrated practices for sample collection, refrigerated storage, freezing, thawing, rules for use and disposal, transfer agreements, and database development. Indeed the development of this new industry continues to be constrained by factors including the technical requirements for scaling-up to commercial operations during the transition from research, and the absence of uniform quality control practices, industry standards, marketing and price structures, and appropriate biosecurity safeguards (e.g., Figure 2).

However, moving forward in the future will involve more than increasing the scale and types of our activities; we will need to change the way we view these technologies and their utility and value forms. For example, cryopreservation is a technology that provides new ways to generate, maintain and distribute genetic resources. These resources represent a bankable form of wealth. For example, as indicated above, within aquatic biomedical models, genetic resources (e.g., newly characterized mutations or phenotypes) are discovered, catalogued, studied, and integrated into "omic" platforms with each step along this pathway increasing the informational value. Practical utility provides additional value when this genetic information can be manipulated and studied in living organisms (e.g., fish). Thus because of tremendous research effort, the genetic resources associated with biomedical model fishes are increasing rapidly in information value, but are increasingly limited in utility value because of the constraints imposed by maintaining these ever-expanding genetic resources as live populations.

Germplasm is another form of wealth that can be viewed simply as the gametes necessary to perform matings, or more expansively as an exchange currency allowing creation, maintenance and transport of the informational and utility values of genetic resources. If we view this as an economic system, the ability to accumulate, store, and catalog germplasm represents a readily transferable form of wealth that is bankable. In our current system we are over-invested in informational value, constrained in utility value, and essentially without investment in exchange currency. This equation can be balanced by taking advantage of the opportunities offered by establishment of large, interactive germplasm repositories to bank genetic resources. To fully open new mechanisms for accruing value from genetic resources, germplasm banking must be on a scale of thousands or tens of thousands of samples This can only be accomplished by development of high-throughput cryopreservation approaches integrating biological variables, cryobiological principles, equipment and facility development, process control for sample handling, inventory and databasing, quality control and assessment, standardization and establishment of industrial standards, and institution of biosecurity systems – in short, more than simply freezing a few sperm samples. This would also involve a conceptual shift from an informational (theoretical) bias to an expansion of utility value by recognizing the essentially unexploited value of germplasm.

As a practical example, the substantial genetic improvement in global dairy herds has been accomplished almost entirely through the use of cryopreserved sperm to enable selective breeding of bulls to serve as a means to improve milk yields in their daughters. This has produced a multi-billion dollar global market for germplasm where the genetic resources (germplasm) are worth more than the individual bulls they originated from. Genetic information (e.g., data on milk production) is converted into utility value (more efficient dairy herds) through cryopreserved germplasm (the exchange currency). Discussions within aquatic species communities are needed to facilitate the transfer of conceptual approaches that have succeeded in organisms such as livestock by looking for linkages across current needs and opportunities in facilities, equipment, and protocols. High-throughput should be scalable to the needs of

individual laboratories and should strive to establish a central pathway that can accommodate all current levels and methods of application, while simultaneously funneling these activities into a standardized approach that incorporates new technologies such as microfluidics and micro-devices (e.g., see new chapter by Park et al.). This process should also take advantage of industrial methods supported by commercial vendors of specialized equipment, supplies and reagents, and industrial-level service providers for cryopreservation, storage and quality control.

Matters such as these have been actively considered in fields outside of aquatic sciences. In fact, books such as *Improving Cattle by the Millions: NAAB and the Development and Worldwide Application of Artificial Insemination* (Herman 1981) should be required reading for all who wish to apply cryopreservation to aquatic species. That book provides a history of the National Association of Animal Breeders (NAAB) and their activities with dairy and beef cattle throughout the 20th century. It is interesting to note (as done in the first edition of this book) that early in the century, arguments against the use of artificial insemination in cattle included:

1) Reaction to the word "artificial" which lead to claims that such work was against the laws of God or nature.
2) The fear that it would produce abnormal offspring.
3) The concern that it would alter sex ratios.
4) The concern that it would spread disease.
5) The concern that mistakes would result in contaminated bloodlines.
6) The concern that it required too much time and effort.
7) The concern that it would cost too much.

It is perhaps significant that as we continue to move into the 21st century we find these same objections directed against the application of cryopreservation to aquatic species. Given that these and other arguments were refuted or easily remedied, it is clear that there is more than technique that can be transferred from livestock to aquatic species.

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I. Gonads and Gametes

Original Chapter: Revised by Authors

Gonads and Gametes of Fishes

Harry Grier and Carole Neidig

Overview

Teleosts that may be cultured as food fish, and whose gametes are worth cryopreserving for aquaculture or genetic conservation, primarily share a common reproductive mode: external fertilization. Gametes are shed into the aqueous, external environment where fertilization and egg development occur. Some of these teleosts are lower fishes (e.g., families Salmonidae, Esocidae, Siluridae and Cyprinidae) and others are higher fishes (e.g., order Perciformes). Differences in gonadal morphology between lower and higher teleosts are minor, but notable. In males and females, gametes are derived from a germinal epithelium (Grier 2000, Parenti and Grier 2004, Grier et al. 2009) that is composed of germ cells and somatic cells. Germinal epithelia rest upon a basement membrane, as do all epithelia, which also separates them from the stromal (females) or interstitial (males) compartments of the ovaries and testes. This is expressed in numerous histology textbooks (see Grier and Lo Nostro 2000) that epithelia rest upon a basement membrane, but the epithelium is considered to be only the epithelial cells.

In females, the germ cells within the germinal epithelium are oogonia and meiotic oocytes; primary oocyte growth commences within the germinal epithelium after meiotic arrest and prior to the completion of folliculogenesis, as in common snook *Centropomus undecimalis* (Grier 2000, Figures 6 and 7). The somatic cells in the germinal epithelium are epithelial cells that become prefollicle cells when associated with germ cells. Finally, they become follicle cells surrounding an oocyte at the completion of folliculogenesis. An ovarian follicle (Grier 2000) is strictly composed of the oocyte and follicle cells; it is an epithelial derivative. The follicle is surrounded by a basement membrane and theca. Further, this definition of an ovarian follicle reflects upon the conserved process of folliculogenesis among fish and mammals. This is the textbook definition of an ovarian follicle (see Grier 2000, Grier and Lo Nostro 2000, Grier et al. 2009) and supports a unified terminology between vertebrates (Grier and Lo Nostro 2000) that is based on homology of biological structure. This definition is at odds with the variable-by-author definitions of an ovarian follicle presented in the fish literature that generally include a basement membrane and always include the theca (see Grier 2000, Grier and Lo Nostro 2000).

The definition of an ovarian follicle is not based on it being a functional unit, in acknowledgment of the physiological role the theca plays in oocyte and follicle development, as in recent reviews (Le Menn et al. 2007, Lubzens et al. 2010). Based on new information, the process of folliculogenesis, the "functional unit," composed of the follicle, basement membrane and theca, has been defined as a "follicle complex" (Grier 2000, Grier and Lo Nostro 2000). As a result of inconsistent terminology in the fish reproductive literature, a tiered staging schema for oocyte development was developed (Grier et al. 2009). Six, stable oocyte stages were defined, beginning with the mitotic division of oogonia and ending with ovulation. Terminology in use was retained and redefined according to new information. Similarly, the same was done for male fish (Grier and Uribe 2009) based on changes in the male germinal epithelium being used to define annual reproductive phases. The new oocyte staging schema, developed for saltwater, perciform fish, has potential to become universal; it has been applied to a freshwater, siluriform catfish the spotted pimelodus *Pimelodus maculatus* (Quagio-Grassiotto et al. unpublished) with

Grier, H. and C. Neidig. 2011. Gonads and Gametes of Fishes. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 19-32.

appropriate modification. The staging schema is potentially universal as its “Stages” are subdivided into “Steps” (Table 1). The “Steps” are eliminated or moved in different positions to suit individual species or systematic groupings of species. For example, the “oil droplets” step was eliminated from the *P. maculatus* staging as has to be done for all ostariophysan fish (i.e., silurids, cyprinids, gymnotids, characids). Also, changes in the steps within the “Stage Oocyte Maturation” were needed. However, the process of folliculogenesis is precisely the same between *P. maculatus* and *C. undecimalis*. Folliculogenesis is a conserved process.

A notable difference between male and female gamete development is that in males numerous, isogenic sperm develop within a spermatocyst whose borders are formed by somatic cells, the Sertoli cells. The spermatocyst equivalent in females is the ovarian follicle that has a single germ cell surrounded by somatic follicle cells. A second distinguishing character of the female germ cell is meiotic arrest, which is conserved throughout the vertebrates. During meiotic arrest, oocyte growth and maturation occur with arrest being broken only at the end of oocyte maturation, just prior to ovulation. Meiosis then proceeds to metaphase of the second meiotic division when a second arrest occurs which lasts until fertilization.

General Organization of the Testis

In males, germ cells are spermatogonia, spermatocytes, spermatids and sperm. In fish, spermatogenesis occurs within spermatocysts, and sperm are released into the lumina of anastomosing tubules (lower fishes) or lobules (higher fishes) (Grier 1993, Parenti and Grier 2004). The difference between anastomosing tubules and lobules is a matter of degree rather than kind, but this is significant because there are also systematic implications in the evolution of testis types in fish. The teleost testis is divided into germinal and interstitial compartments (Callard 1991, Grier 1993), with separate functions of the two compartments reflected by the cell types of which they are composed (Figure 1). The interstitial compartment contains the blood supply to the testis, contractile myoid cells and androgen-secreting Leydig cells (Figure 2B). Myoid cells have the characteristics of smooth muscle cells and form an incomplete layer over the surface of the germinal compartment (Grier 1993). In northern pike *Esox lucius*, the muscle cells are attached to each other via macula densa (Grier et al. 1989). Presumably, their contractions, particularly around the testis ducts, aid in expelling sperm during spawning.

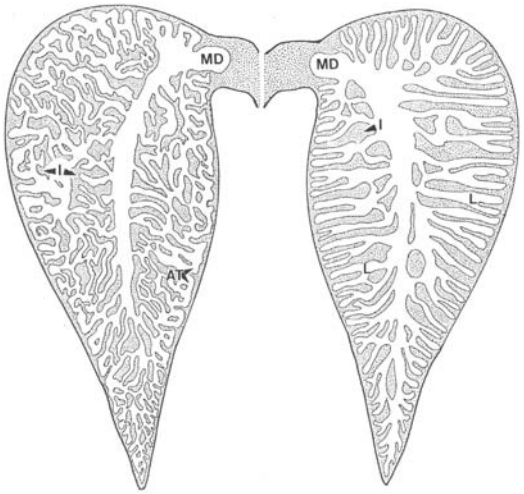

Figure 1. Diagram of anastomosing tubular (left) and lobular (right) fish testes. The interstitial compartments (I) of the testes are stippled while the germinal compartments are white, labeled anastomosing tubules (AT) or branching lobules (L). Lobules terminate at the periphery of the testis. Sperm drain into the main duct (MD). Note that anastomosing of the germinal compartment in the lobular testis type can occur medially, but does not occur at the periphery of the testis.

In the germinal compartment in the fish testis, dual functions of spermatogenesis and sperm storage take place (Grier 1993, Grier and Taylor 1998). As indicated above, germinal compartment morphology in fishes has been used to distinguish two testis types: the anastomosing tubular testis, and the lobular testis. In the anastomosing tubular testis the germinal compartment forms a highly interconnected, anastomosing system of considerable complexity (Figure 1). At the periphery of the testis, the germinal compartment forms loops that double back and unite with the anastomosing network tubules. This testis type has only been observed in lower fishes such as bowfin *Amia calva* and Florida gar *Lepisosteus platyrhinchus* (Holostei), rainbow trout *Oncorhynchus mykiss* (Salmoniformes), northern pike and chain pickerel *Esox niger* (Esociformes) (Grier 1993) and goldfish *Carassius auratus* (Cypriniformes) (Grier personal observation). Many species that have the anastomosing tubular testis type have been domesticated for hundreds of years (goldfish for example) and others are important in aquaculture as commercial food or game fish (e.g., pink salmon *Oncorhynchus gorbuscha*, rainbow trout, and northern pike).

As characteristic of higher fishes (Grier 1993, Parenti and Grier 2004), the germinal compartments are arranged as lobules that terminate blindly at the periphery of the testis (Figure 1). However, lobular testes may also form anastomosing networks, near the ducts. In common snook the testicular lobules branch as they grow in length prior to the spawning season (Grier and Taylor 1998). Subsequently, anastomosing may occur as the juxtaposed, lobule lateral walls join and their lumina become confluent (Grier and Taylor 1998). This process of lobular elongation, branching and formation of anastomoses between lobules leads to the formation of a highly complex germinal compartment. As this occurs, there is also a functional shift from total sperm production to spermatogenesis and sperm storage. It is probable that the process of testicular growth, or recrudescence, prior to the fish being reproductive occurs by the same mechanism in all species of fish with lobular testes. Scant information is available, but this appears to be the case in perciform fishes such as red drum *Sciaenops ocellatus* (Grier et al. 1987), speckled sea trout *Cynoscion nebulosus* (Brown-Peterson et al. 1988) and blue tilapia *Oreochromis aureus* (Grier and Abraham 1983). All of these species are multiple spawners that produce clutches of eggs during the spawning season. In teleosts that spawn only once during a reproductive season, or have a very restricted spawning season, the process of testicular maturation is completed prior to spawning and the entire testis essentially becomes a sperm storage organ, as is the case in the striped mullet *Mugil cephalus* (Grier unpublished).

In either anastomosing tubular or lobular testis types, the germinal epithelium contains two cell types: the Sertoli cells, and the germ cells that give rise to sperm. Most fishes important to aquaculture fertilize eggs externally, and although sperm morphology can vary among taxa, it is generally classified as “teleostean aquasperm” (Figure 2A, next page) (Jamieson 1991). Typically, aquasperm have spherical nuclei, and in scanning electron micrographs, the mid-piece is difficult to distinguish (Figure 2A, B). Generally, there is a single flagellum for motility, but some fish sperm are biflagellated (Mattei 1970, 1991, Jamieson 1991).

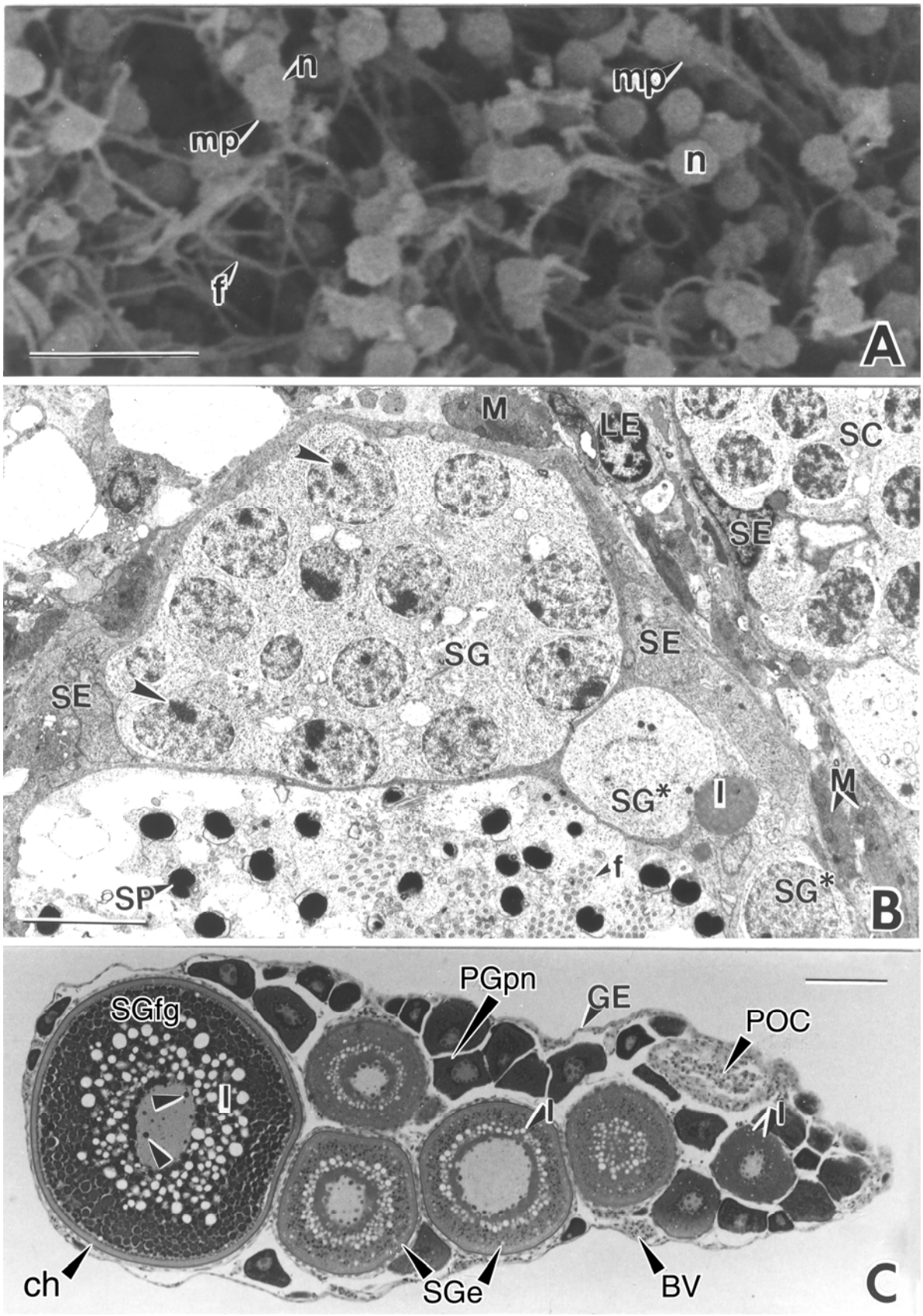
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Figure 2. (Top panel) Scanning electron micrograph showing the basic morphology of teleost aquasperm as exemplified by redbelly tilapia *Tilapia zilli*. The spherical nucleus (n) is <2 μm in diameter; the mid-piece (mp) is nearly indistinct; flagella (f) are seemingly tangled within the main testis duct (bar = 4μm).

(Middle panel) Transmission electron micrograph showing testis structure in striped mullet *Mugil cephalus* typical of fish in general. Leydig cells (LE) and myoid cells (M) are found in the interstitial compartment. The basement membrane that separates the interstitial compartment from the germinal compartment of the testis is not clearly visible in the micrograph. The germinal compartment depicts individual spermatogonia (SG*) and spermatogonia within spermatocysts (SG) enclosed by Sertoli cell (SE) processes. Nuclei of mature sperm (SP) are spherical, and closely associated flagella are present. Sperm reside within the lumen of the lobule. Meiotic spermatocytes (SC) can be distinguished from secondary spermatogonia by the latter still having nucleoli (arrows), which disappear during meiotic prophase. A lipid globule (l) is visible within the Sertoli cell cytoplasm (bar = 5μm).

(Bottom panel) Light micrograph of an ovarian lamella in cross section from common snook. Oocyte staging uses a letter system and updated nomenclature (Grier et al. 2009). The germinal epithelium (GE) lines the periphery of the lamella that borders upon the ovarian lumen or cavity. Previtellogenic, primary growth oocytes have intensely staining basophilic ooplasm and are primarily located beneath the germinal epithelium from which they originated. One primary growth oocyte is in the perinucleolar step of primary growth (PGpn), and small, spherical nucleoli are observed around the periphery of its germinal vesicle. Right to left, oil or lipid droplets (l) begin to appear, initially being somewhat scattered in the ooplasm of a growing oocyte. Developing yolk globules, as small, dense structures, are peripheral to the lipid or oil droplets (l) which encircle the germinal vesicle during early secondary growth (SGe). They are larger and more prominent than previously, but become even larger yet in full-grown oocytes (SGfg). The ooplasm remains "zoned" with oil droplets surrounding the germinal vesicle and are intermixed with dark-staining yolk globules that extend to the oocyte perpiphery. Small, clear vesicles (not labeled) at the oocyte periphery are cortical alveoli, present in all fish oocytes. Higher magnification would reveal that these are intermixed, particularly, with small yolk globules. A secondary growth, full-grown oocyte is approximately 450 μm in diameter. Nucleoli (arrows) tend to remain clustered at the periphery of the nucleus. The oocytes are enclosed by a chorion (ch) or zona pellucida, which is most notable in the full-grown oocyte. A postovulatory follicle complex (POC) is seen, indicating recent ovulation (bar = 100 μm).

Spermatogenesis

Germinal epithelium is unique among epithelia because within this tissue a specialized cell division, called meiosis, occurs. This consists of two successive divisions during which the organismal diploid chromosome number is reduced by half, becoming the haploid germ cell number. In the male germinal epithelium, there is a succession of germ cell stages that begins with spermatogonia and progresses through primary and secondary spermatocytes, spermatids and finally sperm. Spermatogonia are the progenitor diploid cells that divide by mitosis within the germinal epithelium to maintain their presence or enter into meiosis, becoming destined to produce sperm. Primary spermatogonia are surrounded by Sertoli cell processes, whereas secondary spermatogonia are confined to a spermatocyst and will become meiotic primary spermatocytes. The transition of secondary spermatogonia into meiotic primary spermatocytes is marked by the disappearance of the nucleoli (Figure 2B). The cell resulting from the first meiotic

division is a haploid, secondary spermatocyte. Secondary spermatocytes immediately enter into the second meiotic division producing a spermatid.

New spermatids, within a spermatocyst, synchronously begin the process of spermiogenesis. Centrioles migrate to the periphery of the cell, and the centriole located at a right angle to the spermatid outer membrane becomes the basal body that forms the flagellum, the motile apparatus of the mature sperm. As the flagellum elongates, the nucleus begins to change shape as chromosomes condense. A nuclear indentation, or a fossa, generally forms, accepting the two centrioles as the flagellum continues to elongate. The mitochondria enlarge and migrate to surround the proximal end of the flagellum. This orientation of mitochondria and flagellum forms the mid-piece of the mature spermatozoan (Figure 3, next page). Meanwhile, the spermatid cytoplasm also migrates posteriorly and is cast off as the residual body, which is phagocytized by Sertoli cells. Residual body phagocytosis is found in all vertebrate groups and has been proposed to signify homology of Sertoli cells (Grier 1993) among vertebrates.

Within vertebrates, fish are the most reproductively diverse, which is reflected in sperm morphology, particularly in internally fertilizing fishes (Billard 1970, Mattei 1970, 1991, Jamieson 1991, Burns et al. 1995, 2009, Burns and Weitzman 2005). External fertilization is characteristic of most finfish considered to be suitable for aquaculture. And, with few exceptions, aquasperm of all these species have a basic morphology that is surprisingly uniform: all lack an acrosome, all have a rounded nucleus, short mid-piece and a flagellum for motility (Figures 2A, and 3A, B, C). Sperm nuclei are generally about 2 μm in diameter. The flagellum extends from the basal body. Typically, the midpiece consists of spherical mitochondria encircling the proximal portion of the flagellum (Figure 3).

General Organization of the Ovary

The ovaries of lower fishes, such as the salmonids and sturgeons, are suspended from a dorsal mesentery, the mesovarium. The ovary is composed of numerous ovigerous lamellae that project into the coelom forming the gymnovarian ovary. In contrast to lower fishes, the ovaries of higher fishes possess a central lumen into which the ovarian lamellae project. This is the cystovarian ovary. Lamellae consist of stroma in which smooth muscle-like cells and blood vessels occur, in a large extravascular space (Grier et al. 2009). Numerous follicles (an oocyte and surrounding follicle cells) are separated from the stroma by a basement membrane. Outside the basement membrane there is a theca, divided into a theca interna and a theca externa (Grier et al. 2009). The theca interna becomes vascularized during follicle growth while the theca externa borders on the extravascular space in which granulocytes are common.

Fish ovarian lamellae are bounded by an active germinal epithelium in which oogonia and oocytes undergoing folliculogenesis occur (Grier 2000, Parenti and Grier 2004, Grier et al. 2009). Changes in the rate and occurrence of folliculogenesis that are related to fish reproductive modes such as synchronous and group synchronous spawning, are yet to be documented. However, it is known that primary oocyte growth, identified by ooplasmic basophilia (Grier et al. 2009), begins prior to the completion of folliculogenesis in common snook (Grier 2000). Upon ovulation, oocytes become eggs and are shed into the ovarian lumen from where they pass through a gonoduct into the surrounding water during spawning. It is not appropriate to refer to “oviducts” in fish because they lack Müllerian ducts (Uribe et al. 2005). The analogous structure through which eggs pass to the exterior in fish is called a gonoduct.

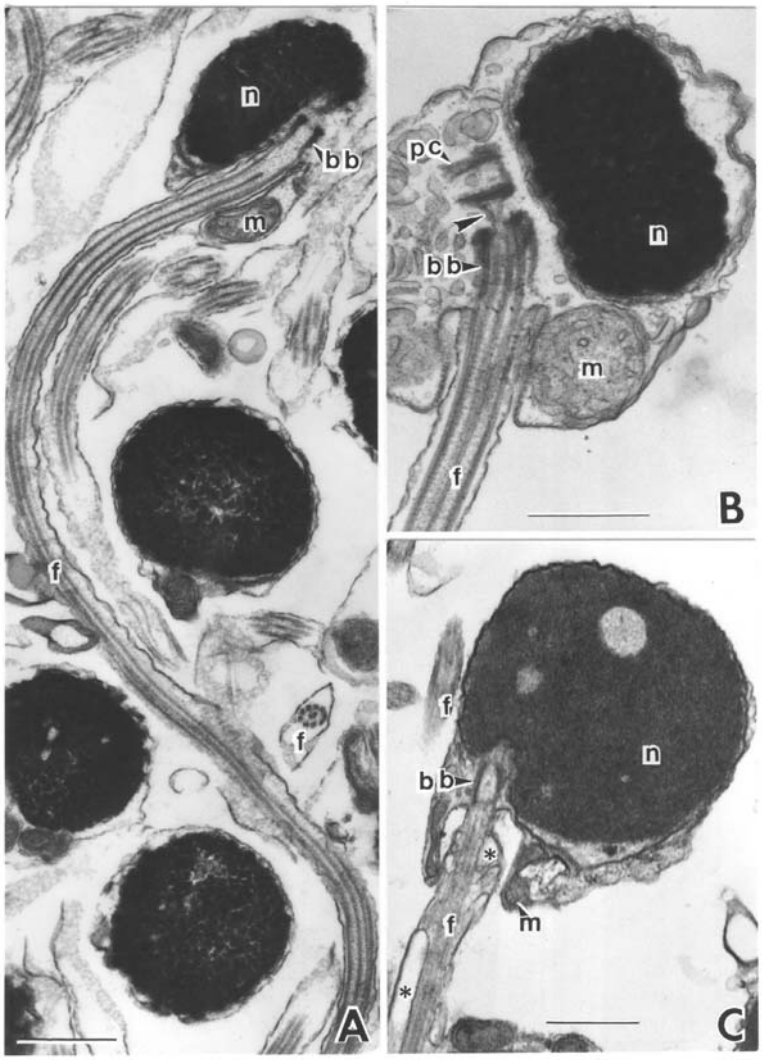

Figure 3. Typical aquasperm from three species of externally fertilizing fishes. (A) Longitudinal section through the sperm of the yellow perch *Perca flavescens* showing the relationship between the nucleus (n), basal body (bb), and flagellum (f) or axoneme. Oblique sections through nearby sperm nuclei reveal that they are circular. This structure is modified by a ventral fossa into which the centriolar complex fits. Bar = 1μm. (B) Longitudinal section through a spermatozoon of the common snook *Centropomus undecimalis*. In cross section, the nucleus is "C"-shaped, but here a longitudinal section is presented. The nucleus surrounds the centriolar complex on three sides. The complex consists of the basal body (bb), which forms the flagellum, and the proximal centriole (pc). The centrioles are connected by electron-dense material (arrow), probably representing structural proteins. Spherical mitochondria (m) form a ring around the flagellum in the mid-piece. Bar = 0.5μm. (C) A nuclear (n) fossa is ventrally present in sperm nuclei of the goldfish *Carassius auratus* and accepts the centriolar complex, of which only the basal body (bb) is seen here. Mid-piece mitochondria (m) are pleomorphic and may branch. These encompass the flagellum (f). Goldfish sperm have the unusual traits of having clear areas in the nucleus that are apparently devoid of condensed DNA and of having vacuolated cytoplasm (*) surrounding the proximal end of the flagellum (f). (Bar = 0.5 μm)

A histological section taken through part of an ovarian lamella in common snook, a multiple-spawning teleost (Figure 2C), reveals follicles with oocytes in various stages of development. Primary oocyte growth commences when basophilia, detected by the common hematoxylin and eosin stain, is observed within growing oocytes. When oocytes in primary growth begin to develop oil droplets and cortical alveoli, the zona pellucida, egg envelope or chorion (terms that are considered to be synonymous) also begins to develop. Secondary oocyte growth, vitellogenesis, is solely the incorporation of yolk globules into an oocyte. It is defined (Wallace and Selman 1990) as the: 1) hepatic synthesis of vitellogenin; 2) delivery of vitellogenin to the oocyte surface via the circulatory system; 3) uptake of vitellogenin by the oocyte via receptor-mediated indocytosis, and 4) translocation of vitellogenin to forming yolk bodies (multivesicular bodies) and its subsequent cleavage into yolk and formation of yolk globules. Vitellogenesis is uniform among the teleosts. It does not include processes that may also be concurrent in a growing oocyte, such as formation of cortical alveoli. Production of cortical alveoli is a separate process that occurs via a different intra-oocyte synthetic pathway than vitellogenesis and involves endoplasmic reticulum and Golgi (Guraya 1986). Sometimes, cortical alveoli develop prior to the appearance of oil droplets in oocytes, as in mummichog, *Fundulus heteroclitus* (Selman and Wallace 1989), or after oil droplets appear, as in common snook (Grier, unpublished), and after the commencement of vitellogenesis, as in European seabass, *Dicentrarchus labrax* (Mayer et al. 1988).

Given the variability in the appearance of cortical alveoli during oocyte growth in teleosts, their positioning as “Steps” in oocyte growth “Stages” can be altered to adapt the tiered staging schema (Grier et al. 2009) to different species of fish. Then, the “Cortical Alveolus Step” may be placed before or after the “Oil Droplets Step” (as in Table 1), or it may be incorporated into the “Early Secondary Growth Step”. Variability in oocyte developmental patterns are easily encompassed by the tiered staging schema in which the frequently used “Cortical Alveolus Stage” (Selman et al. 1986, Begovac and Wallace 1988, Selman and Wallace 1989, Lyman-Gingerich and Pelegri 2007) is subsumed as a final step within the Primary Growth Stage for common snook. Or, it can be moved prior to the Oil Droplets Step for *F. heteroclitus*, or incorporated into secondary growth, as for *D. labrax*. Teleosts are the most reproductive diverse vertebrates, and therefore it should not be expected that a rigid oocyte staging schema would apply to all species. However, the tiered oocyte staging schema (Grier et al. 2009) defines stable “Stages” that are subdivided into flexible “Steps” that render this representation of oocyte development as adaptable. Moreover, the staging schema is the only one that starts at the beginning (mitotic division of oogonia) and finishes at the end (ovulation and formation of a postovulatory follicle complex).

Oocyte development has been typically described using numbering systems, but these vary (West 1990). To circumvent this problem, our hatchery (Neidig et al. 1999) developed a lettering system to describe stages of vitellogenesis in common snook. The letters reflect oocyte development that can be observed in wet-mount biopsies obtained under hatchery conditions using a transmitted-light microscope. The stages were developed so that the aquaculturist could see if oocytes were undergoing maturation prior to ovulation and strip-spawning. However, since the development of this staging, new information has become available necessitating an update of the schema’s nomenclature and adaptation to histological sections of biopsies. As multiple definitions for processes occurring during oocyte development had to be considered, the “tiered staging” is applied here to oocyte development, particularly maturation, in wet mounts (Grier et al. 2009) (Table 1).

In *C. undecimalis*, oocyte stages can be assessed from a small biopsy of oocytes taken from the ovary using a silicon-tubing catheter inserted in the gonoduct oviduct and by applying slight suction. This wet-mount method for oocyte assessment can also be applied to other large fish species that produce pelagic eggs that are about 0.8 mm in diameter. Biopsy samples are placed on a 1 x 3-inch microscope slide, a few drops of water are added, and a coverslip is placed over the sample and slight pressure applied with a blunt instrument or pencil eraser. Pressing down on the coverslip flattens the growing oocytes sufficiently for light to pass through them for evaluation, but the oocytes may burst if too much pressure is applied. Oocytes are examined using a brightfield, compound microscope at 10-x and 40-x magnification. Cortical alveoli cannot be observed within oocytes in wet mounts (Figure 4), and oil droplets are not visible until they began to fuse during maturation, at which time they can be used for interpretation. Oocytes in maturation can be readily discerned, and if a fish is near ovulation the oocytes will be clear. Wet-mount oocyte stages were also verified in histological sections of the biopsies.

The common snook is a saltwater, subtropical species that scatters pelagic eggs containing an oil globule for neutral or slightly positive buoyancy in the water column. Unfortunately, the germinal vesicle is usually displaced to the oocyte periphery when using the wet mount method. This “artifact” should not be taken as a marker for germinal vesicle migration. Germinal vesicle migration can be verified histologically. In view of this, the coalescence of oil droplets to become oil globules (Grier 2009) is much more reliable as an indicator of oocyte development, and can be more easily observed than the germinal vesicle and its migration, because oil globules possess black borders (Figure 4). The coverslip method, originally described by Neidig et al. (2000), allows rapid assessment of oocyte maturation and the timing of spawning without the use noxious fixatives and clearing agents.

Stage PG (primary growth) is the earliest oocyte stage observable in wet mounts. It is divided into five “Steps” that are easily discerned as the oocytes are clear in wet mounts or are well-stained in histological sections. Nucleoli are clearly discerned in primary growth oocytes, but not after vitellogenesis commences and the germinal vesicle appears as a “lighter,” central area in oocytes (Figure 4A). When first formed, the oil/lipid droplets also could not be evaluated in brightfield wet mounts as they were too small or masked by dense, opaque yolk globules. Like cortical alveoli, they can be observed in histological sections. Therefore, histology has been useful in determining that nucleoli were in the perinuclear position throughout vitellogenesis. Subsequently, yolk also began to form as lipid yolk continued to accumulate. When an oocyte has reached maximum diameter, it is full-grown (SGfg) and capable of initiating oocyte maturation (OM) upon appropriate hormonal stimulus. The four “Steps” of OM are OMegv, OMgvm, OMgvb and OMmr (Table 1).

Oocyte Maturation

For aquaculture, reproductive fish can be spawned by three methods: 1) natural spawning; 2) induction of ovulation by hormone application followed by strip spawning, or 3) induction of ovulation by hormone application after which the fish are allowed to spawn naturally. We refer to this latter process as “induced natural spawning”. Before spawning can occur, oocytes must undergo maturation within the follicle and ovulate into the body cavity for (cytoplasmic) and germinal vesicle (nuclear) maturation (Patiño and Sullivan 2002, Grier et al. 2009), not just the migration of the germinal vesicle and its breakdown subsequent to the

resumption of meiosis. Frequently, this is referred to as “final oocyte maturation” or “final maturation,” but these terms are “irrelevant and misleading” (Patiño and Sullivan 2002). They are replaced by “oocyte maturation” or simply “maturation”. The changes taking place during oocyte maturation (Table 1) can be defined by letters (Grier et al. 2009) that have been updated from Neidig et al. (2000).

Table 1. Stages and Steps of oocyte growth and maturation as observed in histological sections and wet-mount preparations of ovarian biopsies. The “Oogonia Proliferate” and “Chromatin Nucleolus” stages have been omitted. Each Stage and Step of oocyte development has a code listed in bold letters. Upper case letters indicate “Stages” while lower case letters indicate “Steps”. Modified after Grier et al. (2009, Science Publishers: SciPub.org).

Growth and maturation stages/steps	Defining characteristics and code: (Stages in upper case letters and steps in lower case letters)
Primary Growth/ one-nucleolus	**PGon:** oocyte is in primary growth (basophilic, blue staining ooplasm in hematoxylin and eosin sections) with one nucleolus.
Primary Growth/ multiple nucleoli	**PGmn:** two or more nucleoli occur within the germinal vesicle (gv).
Primary Growth/ perinucleolar	**PGpn:** multiple nucleoli are arranged around the periphery of the gv. Initially, they are spherical.
Primary Growth/ oil droplets	**PGod:** Oil (lipid) droplets completely encircle the gv. This step in Primary Growth is eliminated for Ostariophysan as oil droplets are lacking.
Primary Growth/ cortical alveolar	**PGca:** cortical alveoli in ooplasm. Initially, they may not be restricted to periphery of the oocyte.
Early Secondary Growth	**SGe:** small yolk globules appear near periphery of the oocyte.
Late Secondary Growth	**SGl:** some yolk globules reach maximum size, but different sized yolk globules are present, smaller ones tend to be between cortical alveoli.
Secondary Growth/full-grown	**SGfg:** Maximum diameter oocyte with a central germinal vesicle (gv) for most species/ooplasm filled with yolk globules/cortical alveoli (not seen in wet mounts) are peripheral beneath the chorion or zona pellucida.
Oocyte Maturation/ eccentric germinal vesicle	**OMegv:** Oil droplets become oil globules as they coalesce around the central gv/yolk not clearing. Oil globules have dark perimeters while the gv perimeter is diffuse in wet mounts. Oil globules coalesce until a single globule exists and gv is displaced to eccentric position. Yolk not clearing.
Oocyte Maturation/ germinal vesicle migration	**OMgvm:** Yolk globules clearing, becoming liquid/gv migration (ooplasm between it and single oil globule).
Oocyte Maturation/ gv breakdown and meiosis resumes	**OMgvb and OMmr:** ooplasm clear, gv breakdown and meiosis resumes, to arrest in metaphase of the second meiotic division/oocyte is preovulatory.
Ovulation	**OV:** Oocytes emerge from the follicle, becoming eggs. Free-flowing eggs. upon slight abdominal compression (hatchery strip spawn) or spawning fish.

Application of Techniques

During ovulation, chemical signals (pheromones) attract males for spawning. The aquaculturist, (insensitive to pheromones), must be diligent in monitoring fish to detect oocyte maturation for spawning when ovulation occurs. If strip spawning is to take place after hormonal

stimulation, it is convenient to be able to monitor the progression of oocyte maturation and ovulation. Depending on the species, there may be a variable time interval during which eggs must be fertilized following ovulation before they become non-viable. Therefore, the culturist must be aware of the biology of the fish being spawned in order to collect samples for the wet-mount technique (Neidig et al. 2000) (Figure 5).

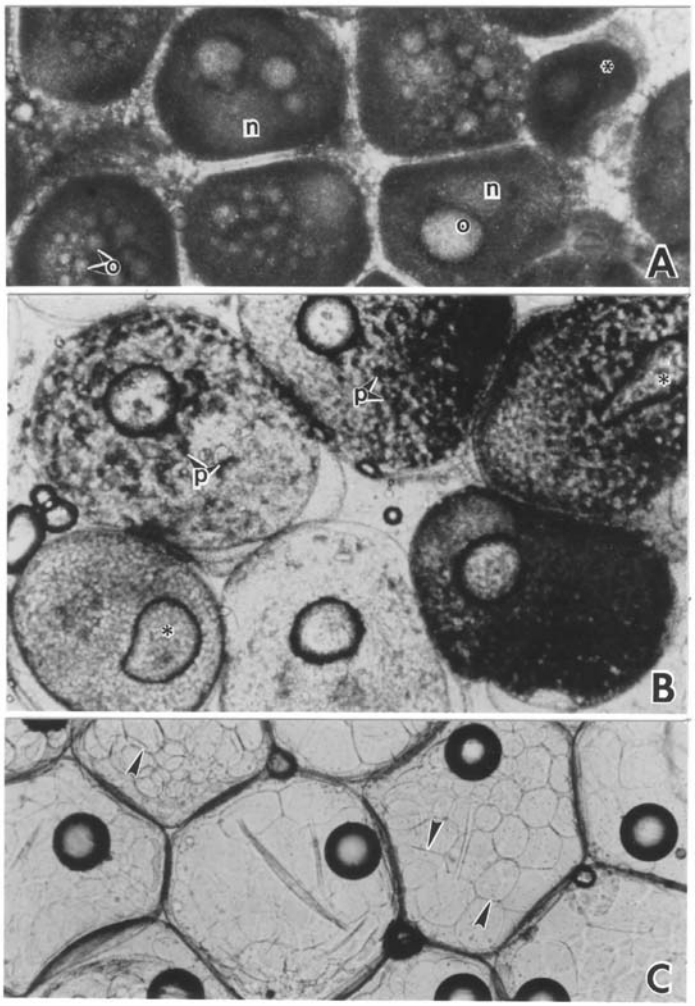

Figure 4. Stages of oocyte maturation illustrated by the coverslip method that has been applied to fresh ovarian biopsies. All photographs were taken at 40-x magnification (size bars were not included as the oocytes were artificially spread to reveal the major cytoplasmic details of maturation used in staging under hatchery conditions).

A) Full-grown and secondary growth oocytes (*) have a uniformly dense ooplasm, generally with a clear, central area indicating the location of the nucleus or germinal vesicle. In OMegv (oocyte maturation - eccentric germinal vesicle step), the first visible event of maturation, is the presence of numerous oil globules (o) that become fewer and larger as maturation progresses. The nucleus (n) in these oocytes is central, but may be peripheral due to its displacement using the wet mount technique. The finality of OMegv is a single oil globule and the germinal vesicle immediately next to it, as in the lower right of the micrograph. The ooplasm is uniformly dense. Oil globules have dark rims whereas the nucleus border is diffuse rendering them excellent for interpretation of maturation.

B) The OMgvm Stage/Step is characterized by clearing ooplasm which is mottled because of protein yolk (p) that has not yet cleared. Early on, there may be multiple oil globules, but they coalesce into a single globule; the nucleus disappears during this stage, entering prophase of the first meiotic division. Oil globules may not be spherical due to the amount of pressure applied to the coverslip. Excessive pressure can cause oocytes to burst; a new sample may have to be examined.

C) Preovulatory oocytes in the OMmr Stage/Steps have a completely clear ooplasm and single oil globule; oocyte diameter is now about 750 μm. Curved lines within the ooplasm (arrows) are characteristic of common snook preovulatory oocytes and eggs and represent oocyte cytoplasm.

Our work centered on common snook which always spawn in the early to late evening (Taylor et al. 1998). Because of this, and as a hatchery protocol, oocyte biopsies were obtained every hour as evening approached on the day following injection with human chorionic gonadotropin (500 IU/Kg) or implantation of time-released pellets of gonadotropin releasing hormone (10 μg per d) (Neidig et al. 2000) until the Stage OMmr (Table 1) was reached. Thereafter, fish were checked at 0.5-hr intervals for ovulation, or freely flowing eggs, using slight abdominal pressure. Upon ovulation, the fish were strip-spawned. As a note of caution, brood fish suffer stress due to handling. Therefore, it must be emphasized that they should be disturbed as little as possible and only then to gain information relative to the hatchery setting.

The wet mount technique was used for real-time evaluation of oocytes undergoing maturation. After an ovarian biopsy was obtained, it was immediately evaluated. In Step OMegv, multiple oil droplets became fusing oil globules that had a dark halo around them (Figure 4A). The oocyte germinal vesicle was a diffuse light area. Oil globules and the germinal vesicle were easily distinguished in fresh biopsies. The finality of the OMegv Stage/Step (Figure 4A), was characterized by the presence of a single oil droplet, without apparent ooplasmic clearing. Induced spawning techniques using hormones can cause the last part of the OMegv Stage/Step to be skipped, as eggs can be released with multiple rather than single oil globules. The normal situation is the formation a single oil globule during ooplasm clearing and germinal vesicle migration (OMgvm Stage/step) (Figure 4B). During this stage, the ooplasm had become translucent but had numerous “blotches” of dark protein yolk globules. In Stage/Step OMmr (the preovulatory oocyte), the ooplasm was completely translucent aside from a reticulated pattern produced by basophilic ooplasm extending between clear yolk globules from the periphery of the oocyte (Figure 4C). This reticulated pattern is not typical in other species of saltwater fish such as red drum in which preovulatory oocytes have a single pool of fluid, hydrated yolk.

Sperm cells are an excellent choice for cryopreservation. They contain little cytoplasm in which ice crystals can form. Aquasperm of mature fish are approximately 2 μm in diameter, and have a rounded nucleus with a basal indentation into which the centriolar complex fits. A few mitochondria surround the proximal end of the flagellum. In at least one species of saltwater fish, the common snook, cryopreserved sperm (stored for 1 yr) had fertilization comparable with fresh sperm when used with eggs from strip-spawned females (Tiersch et al. 2004). Cryopreservation has potential for considerable application in aquaculture and holds promise in sex-changing species, such as serranid fishes. In many protogynous groupers (that naturally change sex from female to male), it is difficult to obtain males, which are always larger and older than females and sometimes inhabit deep water making live capture difficult. If ripe males were captured at sea, enough sperm could be collected and cryopreserved to fertilize numerous spawns at facilities that would be otherwise separated geographically, or by seasonality, from naturally ripe males.

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New Chapter

Spermatogenesis in Fish: A Structural and Functional Basis for Applied Research

Marcelo C. Leal, Rafael H. Nóbrega and Luiz R. França

Introduction

In all groups of vertebrates studied, ranging from fishes to mammals, spermatogenesis proceeds similarly throughout three distinct phases: 1) spermatogonial proliferation, which expands the number of premeiotic cells, 2) meiosis of spermatocytes, and 3) spermiogenesis that involves the differentiation of spermatids into spermatozoa (Russell et al. 1990, Schulz and Miura 2002, Hess and França 2007). This process is dependent on the continuous activity of spermatogonial stem cells, which are the only stem cells in the body that undergo self-renewal throughout life and transmit genetic information to the offspring (Hofmann 2008). Hence, spermatogonial stem cells are considered as the foundation of spermatogenesis and are responsible for sustaining male fertility in vertebrates. Recently, increased knowledge of these cells associated with development of germ cell transplantation techniques has offered great potential value in areas, such as the study of spermatogenesis, production of transgenic animals, cryopreservation of spermatogonial stem cells, preservation of the genetic stocks of valuable animals and endangered species, and in reproductive medicine (Brinster 2007).

In this chapter, we will focus on the basic organization of the germinal epithelium in fishes, morphology of the different types of germ cells, and on some functional aspects of fish spermatogenesis. Also, we address characterization of normal spermatogenesis that would be helpful for diagnosis and study of disturbances of this process that lead to impaired male fertility in fish. Many of the alterations in this process influence spermatozoa structure, such as DNA organization and condensation, flagellum formation, and mitochondrial defects. Therefore, knowledge of the basic aspects and nomenclature of fish spermatogenesis is of fundamental importance for applied research, such as semen cryopreservation.

Organization of the Testis and Spermatogenesis

Similar to other vertebrates, the basic organization of the testis is highly conserved in fishes. The major components of this organization with respect to the following discussion are depicted in Figure 1 (next page). The testis is divided into two major compartments: 1) the tubular compartment composed by the seminiferous epithelium, where the Sertoli cell-germ cell units called cysts are located, the tunica propria composed by the basal lamina and peritubular myoid cells, and the seminiferous tubule lumen filled with fluid secreted by Sertoli cells, and 2) the intertubular compartment containing the steroidogenic Leydig cells, connective tissue cells, and blood vessels (Pudney 1993, Le Gac and Loir 1999). In the seminiferous epithelium, spermatogenesis takes place within cysts or spermatocysts, which are formed when a single spermatogonium is completely surrounded by cytoplasmic projections of Sertoli cells (somatic cells) (Pudney 1993). The cells resulting from differentiating mitotic divisions of this single spermatogonium remain usually interconnected by cytoplasmic bridges that synchronize the development of the members of the same germ cell clone (Pudney 1993, Le Gac and Loir 1999). Thus, in this cystic type of spermatogenesis, a given Sertoli cell is normally in contact with only

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one germ cell type or clone. This is the main difference in relation to the non-cystic spermatogenesis, as occurs in amniote vertebrates (anuran amphibians, reptiles, birds, and mammals), where several clones at different stages of development are distributed along basal, lateral, and adluminal surfaces of a Sertoli cell.

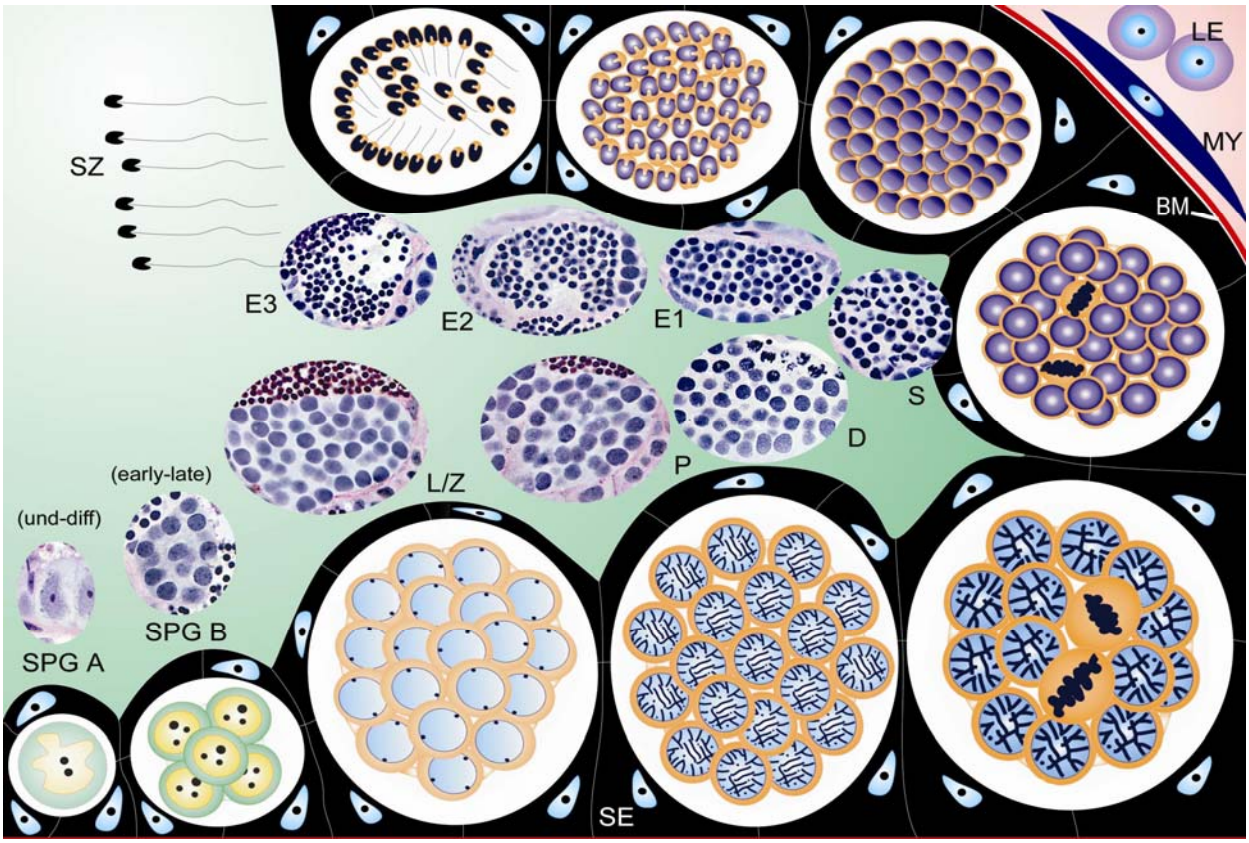

Figure 1. Schematic representation of the spermatogenic process in fish. Nine histological cross sections are shown in the center surrounded by corresponding schematic drawings that serve to depict progressive developmental stages within the germinative compartment of the testis. Spermatogenesis takes place within cysts distributed along the basement membrane (BM) of the seminiferous epithelium. The process begins when cysts are formed by Sertoli cells (SE) completely surrounding type A undifferentiated spermatogonium (lower left of figure). Cells resulting from the incomplete mitosis of the differentiating spermatogonia remain connected by cytoplasmic bridges, developing in a clonal fashion according to the following sequence (proceeding counter-clockwise from lower left): type A undifferentiated spermatogonium to type A differentiated spermatogonia (SPG A) → type B early spermatogonia to type B late spermatogonia (SPG B) → leptotene/zygotene spermatocytes (L/Z) → pachytene spermatocytes (P) → diplotene spermatocytes (D) → secondary spermatocytes (S) → early spermatids (E1) → intermediate spermatids (E2) → late spermatids (E3) → spermatozoa (SZ). Note that in cystic spermatogenesis, a given Sertoli cell is usually in contact with only one germ cell clone. The diagram also illustrates the peritubular myoid cells (MY), and Leydig cells (LE).

Based on recent investigations for the spermatogonial phase (Leal et al. 2009) the following nomenclature is suggested for the progression of fish germ cells during spermatogenesis: primitive/undifferentiated type A spermatogonium give rise to primary/type A differentiated spermatogonia which give rise to secondary/type B spermatogonia (several

generations; the number of generations is species specific) which give rise to preleptotene/leptotene/zygotene spermatocytes which give rise to pachytene spermatocytes which give rise to diplotene spermatocytes which undergo metaphase I resulting in secondary spermatocytes which undergo metaphase II leading to early spermatids, intermediate spermatids, late spermatids and finally, spermatozoa.

Questions and Perspectives

As it occurs in mammals (Russell et al. 1990), it seems that the number of mitotic divisions of the undifferentiated type A spermatogonium, before spermatocytes are formed, changes with phylogenetic relationships. In this regard, stereological investigations have shown that there are different numbers of spermatogonial generations in fish belonging to different orders (Nóbrega et al. 2009). Although there is no reliable marker to distinguish the spermatogonial generations in mammals and fish, nor a consensus concerning the terminology to address the different spermatogonial generations in fish, some criteria such as cell and nuclear size, nuclear morphology and the number of cells per cyst have been used to differentiate early spermatogonia from the more differentiated ones.

The development of germ cells, from spermatogonia to spermatozoa, is strictly regulated, and requires a specific microenvironment that is created by Sertoli cells (Schulz and Miura 2002). In this way, survival depends ultimately on the interactions between Sertoli cells, and between Sertoli cells and germ cells (Batlouni et al. 2009). These interactions are crucial to provide the structural and physiological support necessary for germ cell development during spermatogenesis in fish (Pudney 1993).

As an illustrative aspect we mention that many possible alterations can occur during spermatogenesis (in somatic or germ cell elements), causing reductions in sperm production or quality, and leading eventually to infertility. For instance, it has been reported that abnormal recombination of homologous chromosomes may occur during meiosis (Leal et al. 2007). Also, defects on the spermatid structure may be due to problems during spermiogenesis, and environmental factors such as the presence of toxic substances or endocrine disruptors (e.g. pseudo-estrogens) may affect spermatogonial differentiation (Ortiz-Zarragoitia et al. 2005, Khatchadourian et al. 2007). Therefore, although only briefly overviewed in this chapter, we hope that knowledge of fish spermatogenesis can prompt research on alterations or disturbances regarding the functionality of spermatozoa that take place during the freezing and thawing processes in addition to those that can occur during the spermatogenic process.

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Induced Ovulation and Spermiation, and Factors Influencing Gamete Quality of Fishes

Edward Donaldson, Igor I. Solar and Brian Harvey

Introduction

The recent dramatic worldwide declines in natural fish stocks (New 1997), largely resulting from overexploitation and anthropogenic changes in the environment, have led to increasing interest in the creation of gene banks for wild aquatic organisms (Harvey 1996). Furthermore, the global growth of intensive aquaculture has increased the need for efficient and effective means of conserving fish gametes (Donaldson 1997). The successful conservation and utilization of conserved gametes are dependent on a supply of high quality gametes. Thus, it is a truism that milt quality is never improved by cryopreservation and that the successful utilization of cryopreserved sperm is dependent on the availability of ova of high quality.

Gamete quality is influenced by a number of factors during broodstock maturation, by methods used to induce gamete production, and by methods utilized during gamete collection. In this chapter, we describe current methods for the induction of ovulation and spermiation and several factors that influence gamete quality, and therefore, the ultimate success of the conservation process.

Induced Ovulation and Spermiation

In some species, such as the salmonids (*Oncorhynchus* spp.), ovulation and spermiation occur spontaneously in broodstock held in captivity. In other species where either males or females do not undergo final gamete maturation in culture**,** it is necessary to manipulate the environment (e.g. temperature, photoperiod, or salinity) or utilize endocrine technologies to ensure gamete availability. Even in salmonids, where spontaneous ovulation and spermiation occur in captivity, endocrine technologies are being used to obtain gametes earlier than would normally be possible, to synchronize final maturation ensuring that all ova are ovulated and to maximize milt yield.

Induced ovulation and spermiation technologies, first developed six decades ago, have evolved rapidly in recent yr from the use of simple, gonadotropin-containing, pituitary homogenates (Von Ihering 1937) or partially purified piscine gonadotropin preparations (Donaldson et al. 1972) to the use of potent synthetic analogs of gonadotropin releasing hormone (GnRH) (Donaldson 1996). The gonadotropin(s) present in the traditional pituitary homogenate act directly at the level of the ovary or testis. The use of homologous pituitary preparations or gonadotropins can be very successful, but the species-specific nature of the gonadotropins results in varying success in related species. The quality of pituitary glands can vary depending, for example, on the state of maturation of the donor fish and the methods used to preserve the glands, although procedures have been developed to standardize potency (Yaron et al. 1984, 1985). Furthermore, the cost of good quality pituitary glands has escalated in recent yr.

The gonadotropin releasing hormones act at the level of the pituitary gland by stimulation of the synthesis and release of endogenous gonadotropin(s). Structurally, the nonapeptide analogs of GnRH are derived from the natural GnRH decapeptides. Typically, the potent agonist

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analogs are modified from the original GnRH structure by substitution with an appropriate D amino acid in position 6 and by deletion of the terminal glycine in position 10 and its replacement with an ethylamide. Over a dozen natural GnRH peptides have been identified to date in species ranging from tunicates to mammals, with the greatest variety occurring in the teleosts (Sherwood et al. 1993). Several GnRH analogs derived from the mammalian GnRH (mGnRH) structure have been utilized successfully in fish. These mammalian GnRH analogs were originally (and still can be) referred to as luteinizing hormone releasing hormone analogs (LHRHa). Other GnRH analogs which have been shown to be effective in fish include those derived from chicken *Gallus gallus* II GnRH and salmon *Oncorhynchus* spp. Gonadotropin releasing hormone analogs, which have been successfully used in a wide variety of fish, include the [D-Ala^6, Des, Gly^{10}] LHRH ethylamide and the [D-Arg^6, Des, Gly^{10}] salmon GnRH ethylamide. Thus the [D-Ala^6, Des, Gly^{10}] LHRH ethylamide analog (LHRHa) has recently been utilized to increase milt volume in the Mediterranean sea bass *Dicentrarchus labrax* (Sorbera et al. 1996) (Figure 1).

In many fish species, such as salmonids, LHRHa is effective in inducing ovulation and spermiation, while in some other fish species, especially carps and catfishes, there is significant dopaminergic inhibition of gonadotropin release from the pituitary gland (Peter et al. 1988). In these species, the LHRHa or GnRHa is co-administered with a suitable dopamine antagonist. Dopamine antagonists that have been used successfully in conjunction with LHRHa or GnRHa include pimozide, domperidone (Peter et al. 1988), metoclopromide (Yaron 1995) and sulpiride.

GnRHa or LHRHa can be administered to fish in a variety of ways. Intraperitoneal or intramuscular injection of an aqueous solution is the most common route, but suspensions of LHRHa in lipid and suspensions of microencapsulated LHRHa can also be injected. LHRHa can also be incorporated into controlled-release cholesterol (Crim and Glebe 1994) or polymer (Zohar 1988, Solar et al. 1995) pellets that can be implanted into broodstock. Oral administration of GnRHa in combination with a dopamine antagonist has also been successfully utilized to induce ovulation in the Thai carp *Puntius gonionotus* (Sukumasavin et al. 1992). Finally, ultrasound has been utilized to enhance uptake of LHRHa into fish immersed in a solution of LHRHa.

In the future, we can expect further developments in the control of ovulation and spermiation in fish that are based on the manipulation of feedback control of gonadotropin synthesis and release. Thus, antiestrogens such as tamoxifen have been used in fish to induce ovulation (Donaldson et al. 1981). Recently, we have shown that the aromatase inhibitor fadrozole can inhibit estrogen biosynthesis in the coho salmon *Oncorhynchus kisutch* (Afonso et al. 1997) and aromatase inhibitors may thus provide a further means of inducing ovulation and spermiation in fish.

Factors Influencing Gamete Quality

Males

The quality of milt, and therefore its suitability for cryopreservation, is influenced by a wide variety of factors. Above all, it is important during stripping of milt to avoid contamination with urine, feces, water or mucus. This can be achieved by wiping the male after removal from the water, and by elimination of urine by gentle pressure before collecting milt. Alternatively, the sperm duct can be catheterized with a polyethylene cannula prior to stripping (Rana 1995). The avoidance of contamination is particularly important when the milt is not frozen immediately.

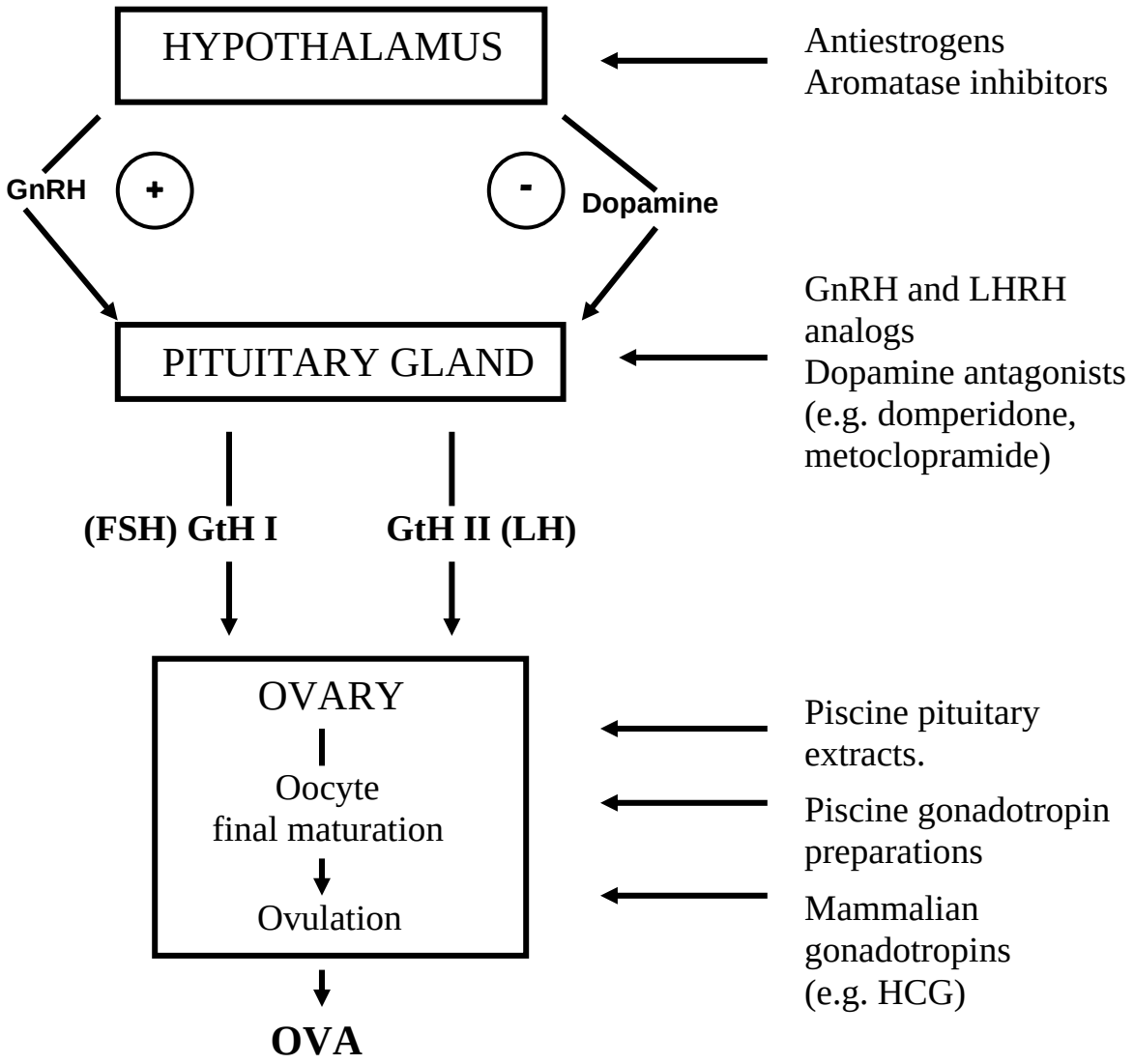

Figure 1. Controlled ovulation by manipulation of the hypothalamic-pituitary-gonadal axis. Modified from Donaldson and Devlin (1996), gonadotropin terminology according to Swanson (1991).

Thus, in Scotland, in studies where milt from Atlantic salmon *Salmo salar* was transported back to the laboratory for cryopreservation, catheterization was used on a regular basis (K. Rana personal communication). On the other hand, we have achieved high fertility rates with regular-stripped milt that was cryopreserved immediately after stripping in the field using a liquid nitrogen dry shipper. If milt must be stored or transported prior to cryopreservation it is important that it be stored under optimal conditions (i.e. in a thin layer to facilitate oxygen diffusion), in a plastic bag, under oxygen, at an appropriate temperature for the species. In salmonids, this would be on ice but protected from direct contact with the ice (Cloud et al. 1990). Where milt is to be stored for an extended period, the addition of low levels of an appropriate antibiotic can lengthen the storage period (Stoss 1983).

There is a shortage of hard experimental data on factors that influence milt quality during broodstock maturation. However, several factors may be of importance including broodstock diet

and time of milt collection during the spawning season. With regard to the latter, there is some evidence (e.g. in rainbow trout *Oncorhynchus mykiss*) that milt quality declines toward the end of the spawning season (Moccia and Munkitrick 1987). This may result from a delay between spermiation and stripping. In anadromous species such as the salmonids, salinity during final maturation can be important. Milt quality can be lower in salmonids from salt water compared to those from fresh water (e.g. in chum salmon *Oncorhynchus keta*) (Stoss and Fagerlund 1982). In addition, elevated rearing temperatures in salmonids during gametogenesis can reduce gamete quality. However, this may be more serious in females than in males.

Females

Pre-stripping factors that can influence egg quality include broodstock diet, rearing temperature, water quality (e.g. salinity), pre-ovulatory stressors, timing of ovulation during the spawning season and time elapsed between ovulation and stripping. In salmonids and other species, diet (e.g. vitamin content, trace mineral content, lipid composition and content) can exert a major influence on egg quality; however, much remains to be learned if cultured fish are to match or exceed the egg quality found in nature. Elevated salinity during final oocyte maturation and ovulation in salmonids can have a negative influence on egg quality. The exposure of rainbow trout females to chronic, intermittent or acute stress during oogenesis can result in lower fertility (Campbell et al. 1992). We have evidence that the stress hormone cortisol, which is synthesized in the interrenal cells, is transferred from the mature female into the oocytes (Stratholt et al. 1997). However, the elevation of cortisol concentration in newly ovulated coho salmon eggs by immersion in cortisol, prior to fertilization, did not reduce survival to hatch (Stratholt et al. 1997). It is generally accepted that egg quality declines in the latter part of the spawning season. This may be caused by a variety of factors including overripening (delay between ovulation and stripping) and deterioration in fish health especially in salmonids that undergo post-spawning mortality.

Post-stripping factors that can influence egg quality and fertility include time between stripping and fertilization and storage conditions (e.g. temperature, oxygen level). The time between stripping and fertilization is a critical factor and ideally, freshly stripped eggs should be used when fertilizing with cryopreserved milt. When eggs are stored, they should be stored at the optimal temperature for the species (just above 0 °C for salmonids). They should also be stored under oxygen no more than three layers deep, to facilitate oxygen diffusion. The addition of antibiotics can extend the storage period as seen in rainbow trout (Stoss and Donaldson 1982).

Field Trial

Recently, a field trial was conducted on the cryopreservation of milt from sockeye salmon *Oncorhynchus nerka* as part of a study on the feasibility of creating a gene bank for Pacific salmon stocks in British Columbia. In this unpublished study, freshly stripped milt was cryopreserved immediately in nitrogen vapor, at a diluent-to-milt ratio of 3:1, in 10-mL straws utilizing a LN_2 dry shipper. The frozen milt was thawed 1 d later and used immediately to fertilize freshly stripped eggs. The ratio of spermatozoa per egg was 4×10^6 sperm per egg (one 10-mL straw per 4,000 eggs). The fertilization rate, evaluated at the eyed stage, was >70% of the control rate (99%) achieved using freshly stripped milt with the same batch of eggs.

Conclusions

It is evident that gamete quality plays an important role in the success of milt cryopreservation and its importance cannot be overemphasized. The field trial indicated that the cryopreservation of fresh milt under field conditions can be used as a basis for the development of a viable gene bank and that similar procedures could also be utilized to conserve high quality, high value milt from aquaculture broodstock.

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Annotated Bibliography of Developments in the Last Decade

Research on gamete quality, induced ovulation, and spermiation in aquatic species has been extremely active since 2000. The following references are representative of the literature available in this area, and include review publications on egg and sperm quality, parent-egg-progeny relationships in teleost fish, effect of stress on reproduction and gamete quality; neuroendocrinology in teleost fish reproduction, including regulation of oocyte maturation in fish, GnRH treatments in finfish reproduction, the use of Ovaprim in ornamental fishes, GnRH in salmoniform and pleuronectiform fishes, and perspectives on gonadotropins in teleosts; broodstock and hormonal manipulations in fish reproduction, including spawning induction in common carp. The remaining references include gamete quality assessment of additional species for which hormonal induction was integral to the study design in hormonal induction for final oocyte maturation, ovulation, and spawning.

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Biochemical Characteristics of Seminal Plasma and Spermatozoa of Freshwater Fishes

Andrzej Ciereszko, Jan Glogowski and Konrad Dabrowski

Introduction

Quality of fresh milt and appropriate cryopreservation techniques are critical to obtain good quality frozen sperm. Appropriate cryopreservation of teleost fish milt should produce sperm with similar structural and functional integrity as that of fresh sperm, including motility, genome packaging, mitochondria and membranes. In the case of acipenserid fishes, which possess a sperm acrosome (Jamieson 1991), integrity and functions of the acrosome must also be preserved. Damage to any one of these components seriously impairs or eliminates the ability of cryopreserved spermatozoa to successfully fertilize the egg. Therefore, there is a need to understand the role of all these components of fish sperm in fertilization to evaluate the conditions before cryopreservation and the impact of cryopreservation on sperm structure and function.

Final quality of fresh milt depends on conditions of spermatogenesis and sperm intratesticular storage. Another critical factor is time of storage of sperm in the spermatic duct. Sperm samples collected at the beginning or end of the spermiation period may be of low quality (Billard and Cosson 1992). The cause of poor sperm quality at the beginning of the spawning season is not known, while a low quality of semen at the end of spermiation may be caused by aging. The usefulness of aged gametes for cryopreservation is low (Billard 1986). Even if the quality of sperm is excellent, often the procedure of milt collection may introduce detrimental contaminants into sperm suspensions. Short-time exposure of sperm to small concentrations of impurities such as urine or feces may have little effect on the fertilizing ability of fresh semen, but may modify sperm characteristics important to successful cryopreservation. Better knowledge of seminal plasma components is important to understand events leading to production of good quality gametes and to identify factors that disturb sperm function.

Semen consists of seminal plasma (or seminal fluid) and spermatozoa. Seminal plasma has a unique composition containing substances supporting sperm cells and some substances reflecting function of the reproductive system and spermatozoa. In this chapter we summarize available data on the composition and possible function of seminal plasma components, including those with proven or potential importance for cryopreservation. We also present those aspects of sperm biochemistry which are related to cryopreservation. Information on fish sperm biology and cryopreservation has been covered in previous reviews (Scott and Baynes 1980, Stoss 1983, Morisawa 1985, 1987, 1994, Billard 1986, 1988, 1990, 1992, Billard and Cosson 1990, 1992, Leung 1991, Leung and Jamieson 1991, Linhart et al. 1991, Piironen 1994, Suquet et al. 1994, Billard et al. 1995a, 1995b, Rana 1995a, 1995b). We attempt to combine this information in a functional manner and point out the areas of investigation requiring further advancement.

Ciereszko, A, J. Glogowski and K. Dabrowski. 2011. Biochemical Characteristics of Seminal Plasma and Spermatozoa of Freshwater Fishes. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 46-79.

Seminal Plasma

The main role of seminal plasma is to create an optimal environment for storage of spermatozoa. Frequently storage may take place for several mo, for example in rainbow trout *Oncorhynchus mykiss* and yellow perch *Perca flavescens* (Billard 1986, 1992, Ciereszko et al. 1998). The conditions of storage should protect sperm fertilizing ability, motility and maintain metabolism to preserve viability and energetic resources for sperm activation (Piironen 1994). In the case of fish with acrosomal spermatozoa, the acrosome must also be preserved. In fish with internal fertilization, constituents of seminal plasma may support spermatozoa during their movement to ova (Yao and Crim 1995). It is also argued that seminal plasma, together with ovarian fluid, have beneficial functions for spermatozoa during external fertilization by creating a favorable micro-environment for sperm movement (Billard 1986). Each of these fluids limit hypo-osmotic shock of sperm or maintain a favorable pH for motility and fertilization.

Seminal fluid in most teleost fishes is a secretory product of the testes and of the spermatic ducts (Loir et al. 1990, Lahnsteiner et al. 1993a, 1994). This is due to the fact that teleost fishes, with some exceptions (suborders Gobioidei and Blennioidae, and *Clarias gariepinus*), have no accessory glands (Ginsburg 1972, Stoss 1983, Van den Hurk et al. 1987, Patzner 1991). Some seminal plasma components are not secretory products, but originate from damaged spermatozoa and cells of the testes or spermatic duct. Disturbances in composition of seminal plasma will lead to impairment of spermatozoa storage and therefore will decrease quality. This will in turn likely result in decreased suitability for cryopreservation.

Mineral Composition of Fish Seminal Plasma

Fish seminal plasma contains mainly mineral compounds and low concentrations of organic substances. Three ions predominate in seminal plasma: sodium, potassium and chloride. Their concentrations range between 75 and 175 mM for sodium, 32 and 86 mM for potassium, and 112 and 183 mM for chloride (Morisawa 1985, Suquet et al. 1994). Calcium and magnesium ions also contribute significantly to ionic composition of seminal plasma, their concentrations are usually in the range of 1 to 2 mM. Although osmolality of seminal plasma is similar to that of blood (Morisawa 1985, Suquet et al. 1994, Koldras et al. 1996), there are significant differences in particular ion concentrations between these body fluids. Generally, potassium concentrations in seminal plasma of freshwater fishes are higher than those in blood plasma. This high potassium concentration in seminal plasma possibly results from the presence of a blood-testis barrier (Viljoen and van Vuren 1992) and absorption of sodium and secretion of potassium by the epithelium of the spermatic duct (Marshall et al. 1989). All these ions are involved in regulation of sperm motility either by their participation in osmolality of seminal plasma or by their direct or modulating effect on sperm activation (Scott and Baynes 1980, Billard and Cosson 1992). Micromolar concentrations of trace metals, such as zinc and boron ions have also been detected in fish seminal plasma, but their role is not certain at present (Schmehl et al. 1987). Zinc ions may have a potential role in the sperm physiology of fish, including phosphorylation of sperm proteins, and involvement in sperm motility and acrosome reactions. Involvement of zinc in these processes was described in the sperm of other aquatic animals (Clapper et al. 1985, Ahluwalia et al. 1991, Morisawa 1994).

Two Mechanisms of Sperm Motility Inhibition by Seminal Plasma Mineral **second** *Substances*

At present, two main mechanisms involved in keeping spermatozoa immotile were described in freshwater fish. The first mechanism, which operates in salmonids, is based on potassium ion concentration. Sperm motility is suppressed by a high concentration of potassium ions and is triggered by a decrease in the concentration of this ion. The mechanism, which operates in other fish species, is based on osmotic pressure. Sperm motility is suppressed by high osmotic pressure (isotonic to seminal plasma) and is triggered by lowered osmotic pressure. Both mechanisms of motility initiation consist of a cascade of events. The details of these cascades are not yet fully understood. Acquisition of potential for sperm motility is accompanied by an increase of pH of seminal plasma due to an increase of bicarbonate concentration (Morisawa and Morisawa 1988, Morisawa et al. 1993, Ohta et al. 1997). Initiation of sperm motility may also be modulated by substances (peptides or proteins) originating from fish eggs (Morisawa 1994).

The concentration of potassium ion in salmonid seminal plasma is in the range of 25 to 40 mM (Morisawa 1985, Suquet et al. 1994). Potassium ion at a concentration of 40 mM is used for extension of salmonid milt to obtain sperm suspensions with non-activated spermatozoa. This procedure has also been employed for motility studies of rainbow trout sperm (Billard and Cosson 1992). The central role of potassium ions in regulation of sperm motility in salmonids has been well established and confirmed in numerous studies (Billard 1992). However, Billard and Cosson (1992) reported that at the beginning and the end of spermiation of rainbow trout, potassium ions were not effective for motility inhibition. It is likely that this ion does not act alone in the control of motility. It is well known that other ions including sodium, calcium and magnesium may interact with the potassium ion. For example, potassium inhibition can be overcome by sodium, calcium and magnesium ions (Billard and Cosson 1992). At present, the molecular mechanisms for interrelationships among these ions have not been elucidated.

The general model of the mechanism of sperm motility initiation in salmonids includes potassium efflux and an increase in intracellular calcium. This ion movement triggers a rise in intraspermatozoal cAMP within 1 sec (Morisawa and Ishida 1987) and subsequent phosphorylation of a protein with a molecular mass of 15 kDa (Hayashi et al. 1987, Jin et al. 1994). Target sites for this protein are located in the basal region of the flagellum (Morisawa 1994). This suggests that interaction of phosphorylated 15 kDa protein with the flagellum may have a direct effect on initiation of sperm movement.

The potassium ion in cyprinid seminal plasma, unlike in salmonids, has no specific role in keeping spermatozoa in the quiescent state. In fact, it can increase the velocity and frequency of sperm motility of common carp *Cyprinus carpio* (Billard and Cosson 1992) and other fish (Lin and Dabrowski 1996). It is currently believed that osmotic pressure is critical to keep sperm immotile in cyprinid fishes. The mechanism of activation of cyprinid spermatozoa is not well understood and no cellular sec messengers have been identified (Perchec Poupard et al. 1997). It has been suggested that potassium and chloride ions play a central role in the perception or transduction of the osmotic signal for activation (Krasznai et al. 1995, Perchec Poupard et al. 1997).

Strong inhibition of sperm motility by potassium ions has also been shown in acrosomal spermatozoa of lake sturgeon (*Acipenser baeri* and *A. fulvescens)* and paddlefish *Polyodon spathula* (Gallis et al. 1991, Cosson and Linhart 1996, Toth et al. 1997). This suggests that potassium ions have a critical role in keeping spermatozoa of acipenserid fishes in a quiescent state, similar to salmonids. It seems however, that the mechanism of sperm motility regulation based on osmotic pressure may operate in acrosomal spermatozoa of sea lamprey *Petromyzon*

marinus (Ciereszko et al. unpublished data) and Arctic lamprey *Lethenteron japonicum* (Kobayashi 1993) where no inhibitory effect of potassium ions was observed for sperm motility.

The mechanism of control of sperm motility may be modified by cryopreservation. Dimethyl sulfoxide (DMSO) abolished potassium ion inhibition of motility in rainbow trout spermatozoa (Scott and Baynes 1980). It was also observed that DMSO could trigger sperm motility in common carp (Perchec-Poupard et al. 1997). Mechanisms of this action of DMSO on spermatozoa motility are unknown. This action may be in part responsible for the poor capacity of motility initiation in cryopreserved common carp (Perchec-Poupard et al. 1997). Recent data have confirmed the low usefulness of DMSO in cryopreservation of common carp semen (Babiak et al. 1997). It was found that dimethyl acetamide (DMA) was clearly superior to DMSO.

Application of Seminal Plasma Mimicking Solutions as Semen Extenders for Cryopreservation

The first step of cryopreservation is the extension of cooled milt with solutions containing cryoprotectants (or the cryoprotectant is added later) to allow the cryoprotectant to penetrate into sperm cells before freezing. For successful cryopreservation, it is critical to prevent any activation of spermatozoa during this step. Two approaches have been used to develop the composition of extenders. The first approach involves a formulation of complex extenders mimicking the composition of seminal plasma, or use of physiological salt solutions developed for fish (Stoss 1983, Erdhal 1986, Erdhal and Graham 1987, Rana et al.1990). The sec approach involves the formulation of simplified extenders. In some cases, (e.g. salmonids), complex and simple extenders each produce similar cryopreservation success (Stoss 1983). At present, due to the trial and error nature of most cryopreservation attempts of fish semen, there are no clear criteria related to sperm physiology concerning the choice of simple or complex extenders for cryopreservation of milt for a particular species.

In some experiments, extenders mimicking the physiological solutions of fish have been proven to be clearly superior to simple extenders as seen in sperm cryopreservation of northern pike *Esox lucius* (Babiak et al. 1995). Extenders inhibiting sperm motility are especially justified when milt cannot be obtained by stripping, such as in the case of channel catfish *Ictalurus punctatus* (Tiersch et al. 1994, Christensen and Tiersch 1997) where a modified Hanks' balanced salt solution (HBSS), was used for extraction of spermatozoa from homogenized testis and as an extender. The use of HBSS also allowed refrigerated storage of thawed semen. A similar approach was used by for European catfish *Silurus glanis* (Linhart et al. 1993). In this study, milt was stripped into an immobilizing solution that later served as an extender. Immobilizing solutions may diminish the deleterious effect of urine contamination, as suggested for short-term sperm storage of turbot *Scophthalmus maximus* (Chereguini et al. 1997). Use of balanced salt solutions may also be beneficial when prolonged equilibration time, allowing better penetration of cryoprotectant, is employed for cryopreservation. In such a case, balanced salt solutions may protect sperm during the equilibration step.

Simple extenders, very often consisting of sugar and cryoprotectant, have been successfully employed for cryopreservation of fish semen. For example, an extender consisting of 0.6 M sucrose and 10% DMSO has been used for rainbow trout (Holtz 1993, Ciereszko and Dabrowski 1996), an extender consisting of 0.3 M glucose and 20% glycerol has been used for whitefish *Coregonus muksun* (Piironen and Hyvarinen 1983, Piironen 1987), and an extender consisting of 0.3 M glucose and 10% DMSO for amago salmon *Oncorhynchus rhodurus* and masu salmon *Oncorhynchus masou* (Ohta et al. 1995). Simple extenders (frequently

supplemented with egg yolk) were used for brown trout *Salmo trutta* and Arctic charr *Salvelinus alpinus* (Piironen 1993). Solutions mimicking fish body fluids can contain several compounds. For example, the fish extender (#6) used by Erdhal (1986) contained nine substances plus the cryoprotectant. For this reason, these are costly and their preparation is time-consuming. Therefore, simple extenders are recommended for use whenever possible. A method is often worth reevaluating if complex extenders can be replaced by simple extenders. Reevaluation their method of cryopreservation in northern pike milt (Glogowski et al. 1997b) found that a simple extender consisting of 0.6 M sucrose, 15% DMSO and 10% egg yolk could replace a previously used complex extender (Babiak et al. 1995).

It has recently been suggested that during cryopreservation the ATP stores necessary for activation of sperm cells could be seriously diminished (Billard et al. 1995a). This may be one of the reasons why cryopreserved spermatozoa have lower quality than fresh samples. One proposed strategy to reverse this problem has been to oxygenate thawed sperm suspensions (Tsvetkova et al. 1995). Oxygenation was also shown to restore freezing resistance of stored milt (Magyary et al. 1996). Recovery of ATP stores of cryopreserved sperm may be critical to future research directed to increase the yield of cryopreservation (Billard et al. 1995a). We share this point of view and think that it may be beneficial to test the usefulness of sperm extenders by studying their effects on preservation of the ATP pool in spermatozoa. The preserving effects may take place as early as during extension of semen before freezing, because damage was detected to sperm cells during this step of cryopreservation (Lahnsteiner et al. 1992b).

Low Molecular Weight Organic Substances

Hormones and Pheromones

The most important steroid hormones in fish seminal plasma seem to be testosterone (T) and 11-ketotestosterone (11-KT) produced by Leydig cells (Loir 1990), and 17α-hydroxy, 20β-dihydroprogesterone (17,20P) produced mainly by spermatozoa (Sakai et al. 1989) and spermatogenic cells (Vizziano et al. 1996). The spermatic duct epithelium may also be involved in the secretion of steroids (Schulz 1986, Lahnsteiner et al. 1993a). Seminal plasma concentrations of steroid hormones were related to season (Baynes and Scott 1985, Koldras et al. 1996), thus they may be used for monitoring the progress of spermatogenesis. In masu salmon, 17,20P may have a critical role in the acquisition of sperm motility (Miura et al. 1992). Its action is mediated through an increase in spermatic duct pH caused by bicarbonate, which in turn increases cAMP content in spermatozoa (Morisawa and Morisawa 1988).

Steroid glucuronides, especially 5β-pregnane-3α-17α-diol-20-one-glucuronide have been identified in semen of African catfish *Clarias gariepinus* and zebrafish *Danio rerio*. They are synthesized in the testis (by Leydig cells) in both species, but additionally in African catfish they are produced by seminal vesicles (Van den Hurk and Resink 1992). These steroids function as pheromones in stimulating ovulation in females. Prostaglandins have also been identified in milt and the possibility of their production by fish spermatozoa has been postulated (Labbe et al. 1995).

Cholesterol

Cholesterol has been identified in the seminal plasma of freshwater fish (De Kruger et al. 1984, Billard et al. 1995a, Lahnsteiner et al. 1995b). Its role may be linked either to steroid hormone biosynthesis (Lahnsteiner et al. 1995b) or to its incorporation into cholesterol esters of

sperm membranes. However, there are no available experimental data supporting these suggestions.

Glycerol

Glycerol has been detected in the seminal plasma of six teleost fish species (Piironen and Hyvarinen 1983). These authors explained its presence in seminal plasma, by analogy to mammals, as an indicator of high lipolytic activity in the testis. Glycerol concentration was highest in sperm of whitefish and lowest in rainbow trout. It is intriguing that glycerol, which is usually a poor cryoprotectant for fish semen (Scott and Baynes 1980, Stoss 1983, Ciereszko et al. 1993), is effective for cryopreservation of whitefish milt (Piironen and Hyvarinen 1983, Piironen 1987). High concentrations of glycerol in whitefish milt were related with its high applicability as a cryoprotective agent for sperm of this species (Piironen and Hyvarinen 1983). It is worth noting that glycerol is produced in high concentrations (up to 0.4 M) by some marine arctic fishes and has a role in their biology as a colligative antifreeze (Raymond 1992). It is tempting to speculate that this cryoprotectant may be especially effective for cryopreservation of sperm in these species.

Vitamins

Ascorbic acid (vitamin C) has been found in the seminal plasma of teleost fish (Ciereszko and Dabrowski 1994). Its concentration in seminal plasma (20 to 40 mg/L) is 4- to 8- fold higher than in blood. Teleost fishes, like humans, nonhuman primates, bats and guinea pigs, cannot synthesize ascorbic acid, because they lack L-gulonolactone oxidase, an enzyme of the ascorbic acid biosynthesis pathway (Dabrowski 1990). This enzyme is present in the kidney of chondrosteans, therefore acipenserid fishes can synthesize vitamin C (Dabrowski 1994). Ascorbic acid in seminal plasma of teleost fishes originates from the diet and its concentration in this fluid can be regulated by dietary treatment (Ciereszko and Dabrowski 1995). It is protected from oxidative damage by high molecular weight substances from seminal plasma, most likely proteins (Liu et al. 1995). Low levels of vitamin C in rainbow trout seminal plasma are associated with poor quality of spermatozoa, including reduced sperm concentration, motility, and fertilizing ability (Ciereszko and Dabrowski 1995, Dabrowski and Ciereszko 1996). Other signs of vitamin C deficiency included disturbances in seminal plasma composition, such as a decrease in protein concentration and anti-proteinase activity in seminal plasma (Ciereszko et al. 1996b). These results suggest that ascorbic acid is involved in optimal functioning of the male reproductive system. The mechanism of vitamin C action on male reproduction of fishes is not known, but it is possible that its anti-oxidative action may be critical for protection of spermatozoa from oxidative damage. Such a mechanism has been found for mammalian spermatozoa (Fraga et al. 1991). It has also been shown in mammals that ascorbic acid may be important for protection of sperm membrane integrity (Aurich et al. 1997) and for survival of sperm after cryopreservation (Beconi et al. 1993).

The presence of other vitamins in semen is not well documented. Our observation suggests that it is possible that carotenoids can be transferred from diets to milt (see below). The presence of carotenoids in rainbow trout milt was reported (Czeczuga 1975, Storebakken and No 1992), but the relationship of this provitamin of vitamin A to semen biology is not known.

Free Amino Acids

Both protein-bound and non-protein free amino acids have been detected in seminal plasma of Euroasian perch *Perca fluviatilis* and salmonids (Schmehl et al. 1987, Linhart et al. 1991, Lahnsteiner et al. 1993a, 1994, 1995b). There are substantial differences between blood and seminal plasma free amino acids (Billard and Menezo 1984). The role of free amino acids in sperm physiology is unclear. They may contribute to seminal plasma osmolality (Billard and Menezo 1984) and have a positive role in sperm vitality (Lahnsteiner et al. 1994). Differences in amino acid composition in seminal plasma and in comparison of seminal plasma and blood plasma amino acids between rainbow trout and common carp have been shown (Billard and Menezo 1984). These authors related the above differences between these species to sperm physiology (duration of sperm motility, shorter for rainbow trout and longer for common carp, as well as to the mechanism of motility regulation) and the function of the reproductive system (constant presence of spermatozoa with good fertilizing ability throughout the year in common carp and a rapid decline of sperm quality at the end of spermiation in rainbow trout).

Sugars

No polysaccharides have been found in seminal plasma of fishes (Lahnsteiner et al. 1992a). However monosaccharides, mainly hexoses such as fructose, galactose, and glucose have been identified in this fluid. Glucose concentrations are usually higher than those of the other two hexoses (Piironen and Hyvarinen 1983, Billard et al. 1995a, Lahnsteiner et al. 1995b). Additionally, xylose has been found in seminal plasma of cyprinids (Lahnsteiner et al. 1994). In mammals, fructose and glucose are used as an energy source by spermatozoa (Mann and Lutwak-Mann 1981). The importance of these sugars in fish semen is not clear. Their presence in seminal plasma has been connected to the high energy demand of the testes during spermatogenesis or for lipid synthesis of spermatozoa (Piironen and Hyvarinen 1983, Soengas et al. 1993). Monosaccharides may be secreted by the spermatic duct (Lahnsteiner et al. 1992a). Hexoses may accumulate in spermatozoa during storage in the spermatic duct and maybe later used as energy sources for motility through generation of ATP via glycolysis (Lahnsteiner et al. 1993b). The occurrence of this pathway is probably indicated by the presence of lactate in seminal plasma (Lahnsteiner et al. 1995b). Likely, seminal plasma monosaccharides cannot be used after ejaculation by sperm cells in the conditions prevalent with external fertilization. However, in the case of fish with internal fertilization, seminal plasma sugars supplemented with nutrients present in the fluids of the female reproductive tract could possibly be used after ejaculation (Stoss 1983).

Citric Acid

Citric acid was found in seminal plasma of freshwater fishes (Baynes et al. 1981, Piironen and Hyvarinen 1983). The concentration of citric acid is strongly correlated with sperm concentration, which may indicate its relationship to sperm physiology (Piironen and Hyvarinen 1983, Piironen 1985). Citric acid may also contribute to osmolality of seminal plasma (Piironen 1994). It has been suggested that citric acid, due to its ability to chelate calcium and magnesium ions in seminal plasma, may be an important part of the mechanism responsible for keeping spermatozoa in the quiescent state (Scott and Baynes 1980, Baynes et al. 1981).

Other Organic Substances

Organic acids such as lactate, pyruvate, ketoglutarate, malate, isocitrate, and oxaloacetate, have been identified in fish seminal plasma (Gosh 1985, Linhart et al. 1991, Billard et al. 1995a). These substances (along with citrate) are metabolites of glycolysis or the Krebs cycle. Possibly their presence, together with oxidoreductase coenzymes, NAD(H) and NADP(H), reflects metabolic activity of spermatozoa or the testes and spermatic duct. The involvement of spermatozoa in metabolism of the organic compounds and coenzymes was indicated by their changes in the spermatozoa and seminal plasma of common carp after 24 hr of storage (Gosh 1985).

Large Molecular Weight Organic Substances

Lipids

Various lipid classes have been found in seminal plasma and their levels are highly variable among fish species, for example from 0.007 g/L for Arctic charr *Salvelinus alpinus* to 1 g/L for Euroasian perch (Piironen and Hyvarinen 1983, Piironen 1994). Low levels of triglycerides were found in seminal plasma of cyprinids (Lahnsteiner et al. 1994). As opposed to salmonids, this was associated with a lack of enzyme systems in cyprinid fish sperm to catabolise triglycerides and a lack of secretion of lipids by the spermatic duct. Seminal plasma lipids were associated with metabolism in spermatozoa (Piironen 1994) and with maintaining integrity of lipid composition of sperm membranes (Loir et al. 1990).

Proteins

Protein concentrations in seminal plasma of most fishes are generally low (<2 g/L) with few exceptions. However, in turbot *Scophthalmus maximus*, for example, a mean concentration of 8.8 g/L was established (Suquet et al. 1993, 1994). Protein concentrations in fish seminal plasma are lower than in mammals (Mann and Lutwak-Mann 1981). Fish seminal plasma proteins may have a multiple source of origin. Immunological methods have identified many similar proteins in blood and seminal plasma of rainbow trout (Loir et al. 1990). Several serum-like proteins were bound to spermatozoa. It has yet to be established if the serum-like proteins of seminal plasma are derived directly from blood or are produced in the reproductive system. The spermatic duct has been found to secrete proteins (Lahnsteiner et al. 1993a). A general protective role toward spermatozoa has been postulated for seminal plasma proteins (Billard 1983).

There are not many data available concerning characterization of seminal plasma proteins. At present, the only two protein groups distinguished in this fluid have been lipoproteins and anti-proteinases. These proteins can be visualized and quantified by electrophoretic methods. Other seminal plasma proteins that have been described are enzymes, and these have only been characterized by their activities.

Lipoproteins

Lipoproteins have been identified in rainbow trout seminal plasma at concentrations of 32.9 $\pm$ 9.5 mg/L (Loir et al. 1990). They were identified as being high density-like (HDL-like) lipoproteins, because they co-migrated with serum HDL during electrophoresis. At least six lipoproteins were identified in seminal plasma after electrophoresis. Among these proteins, two of them with molecular masses 14.5 kDa and 29.0 kDa predominated. Both these lipoproteins were observed to be bound with spermatozoa. It was proposed that seminal lipoproteins

interacted with the sperm plasma membrane to maintain lipid composition during storage in the spermatic duct (Loir et al. 1990). It is possible that this may be important for spermatozoal survival through freezing and thawing, because it is well known that optimal lipid arrangement in sperm membranes is important for successful cryopreservation (Parks 1997).

Anti-proteinases

Anti-proteinase activity has been detected in seminal plasma of teleost fishes and preliminary data suggest that several proteins might contribute to this activity (Dabrowski and Ciereszko 1994). Our recent results concerning separation of seminal plasma proteins by use of chromatography and electrophoresis (Figures 1 and 2) confirmed that there were at least five proteins with anti-trypsin activity in rainbow trout seminal plasma (Ciereszko et al. unpublished data). Most of these had high molecular weights and probably could be classified as serine proteinase inhibitors (serpins). Three of these proteins could be visualized after electrophoresis with the use of Atlantic cod trypsin as a target serine proteinase, and only one could be visualized with the use of bovine trypsin (Figure 2). These proteins may be related to blood plasma proteins because they co-migrated with similar serum proteins (Ciereszko et al. unpublished data). These proteins could also be visualized with Coomasie brilliant blue protein staining, which suggests that they are one of the main proteins of seminal plasma. Other seminal anti-proteinases were detected using ion-exchange chromatography. Recently, a proteinase inhibitor of the serpin family was identified in common carp seminal plasma with a molecular mass of 62 kDa, which resembled mammalian α_1-antiproteinase (Huang et al. 1995). At present, it is not known if this protein occurs in other teleost fish species. Thus it is now clear that there is a group of quantitatively important proteins with anti-proteinase activities in fish seminal plasma.

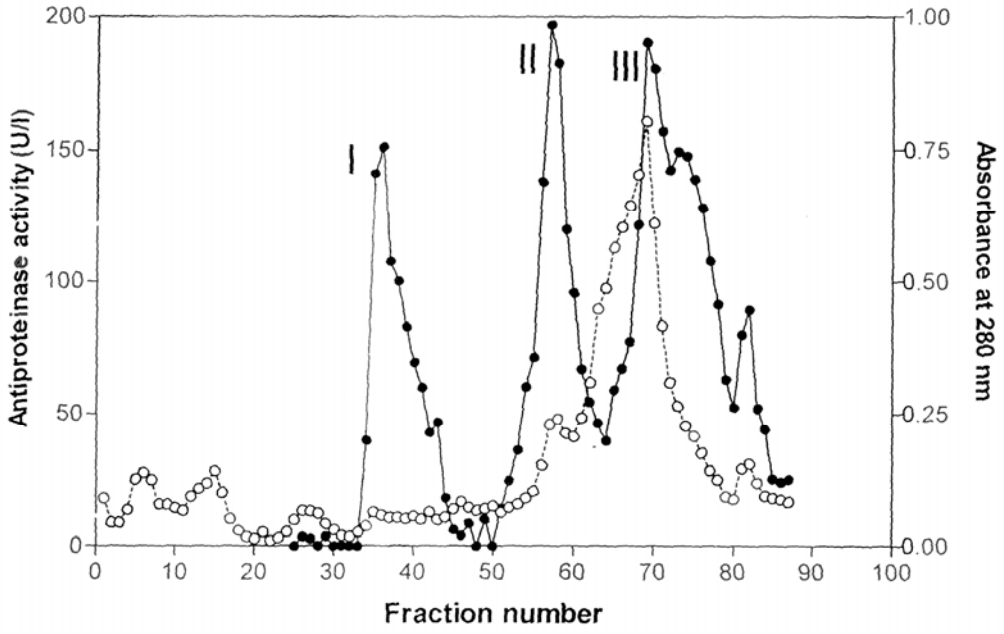

Figure 1. Ion-exchange chromatography of rainbow trout seminal plasma. Three antiproteinase fractions (I-III) are seen when cod trypsin is used for identification (closed circles). The first two fractions can be visualized electrophoretically (See Figure 2).

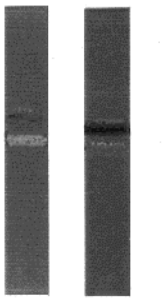

Figure 2. Electrophoretic profiles of antiproteinases of seminal plasma of rainbow trout (white bands) with use of bovine trypsin (left) and cod trypsin (right). Note non-specific esterase (dark band).

The role of serine proteinase inhibitors of seminal plasma is not known, but they likely participate either in protection of spermatozoa from proteolytic attack or in control of physiological events in semen where proteolysis is involved. Protection of spermatozoa may be important for successful storage in the spermatic duct. This suggestion is supported by the observation that anti-trypsin activity (as well as protein concentration) in rainbow trout seminal plasma decreased at the end of the reproductive season (Ciereszko et al. 1996b). It was shown for Mediterranean sea bass *Dicentrarchus labrax*, that at the end of the season a decrease in sperm motility occurred as well as sperm fitness for storage and cryopreservation (Billard 1986). A decrease of anti-proteinase activity toward the end of the reproductive season seems to parallel oncoming events leading to proteolytic destruction of spermatozoa by macrophages or Sertoli cells (Billard and Takashima 1983, Lahnsteiner et al. 1993a). If this assumption is correct, it implicates control by seminal plasma serpins, the serine proteinases of phagocytic cells.

At present, knowledge about the potential physiological role of proteolytic events in teleost fish sperm is not clear. But during recent years some evidence has accumulated suggesting that sperm motility may be regulated in this manner. It has been found that sperm motility may be inhibited by some serine proteinase inhibitors (Cosson and Gagnon 1988, Inaba and Morisawa 1991). Moreover, chymotrypsin-like and trypsin-like proteinases were purified from sperm of chum salmon *Oncorhynchus keta* and are found to be localized along sperm flagella (Inaba and Morisawa 1992, Inaba et al. 1993). If seminal plasma proteinase inhibitors could interact with these proteinases, it would suggest that these inhibitors could be involved in maintaining sperm cells in a quiescent state. If so, it would be an additional regulatory mechanism, in addition to high osmotic pressure or potassium ion concentration, to maintain sperm immotility. This corresponds to the postulated presence of a peptide with a role in keeping spermatozoa in a quiescent state in seminal plasma of carp (Billard 1986). However, until now such a protein or peptide has not been isolated or characterized.

The role of proteolytic activity in seminal plasma is not well understood. The presence of proteolytic activity (measured by azocoll hydrolysis) has been described for seminal plasma of teleost fishes (Lahnsteiner et al. 1993a, 1995b). However, further characterization of this activity

is necessary to evaluate whether it is inhibited by seminal plasma anti-proteinases. It is unlikely that this proteolytic activity is related to trypsin-like proteinase, because no trypsin-like activity was found, at least in free form in seminal plasma of teleost fish (Ciereszko at al. unpublished data). This confirms earlier studies of that were unable to detect trypsin-like activity in seminal plasma (Breton et al. 1974). Miltpain, a new cysteine proteinase has been described from the milt of chum salmon (Kawabata and Ichishima 1997). At present, there are no data on the presence of natural inhibitors of this enzyme in fish semen.

Trypsin-like activity in spermatozoa and anti-trypsin activity in seminal plasma have been found in semen of lake sturgeon and paddlefish (Ciereszko et al. 1994, 1996a). In mammals, a trypsin-like enzyme, acrosin, is located in the acrosome and involved in the acrosome reaction and penetration of the egg envelope (De Jonge et al. 1989). The acrosome reaction has been described in lake sturgeon spermatozoa (Cherr and Clark 1984), but no penetration of the egg envelopes was observed due to the presence of micropyles in eggs. Trypsin-like activity in lake sturgeon and paddlefish spermatozoa resembles the mammalian acrosin. It is possible that proteinase inhibitors of sturgeon seminal plasma may control this enzyme, as seen in mammals. The anti-proteinase inhibitors of seminal plasma in fish with acrosomal spermatozoa may be involved in the control of the acrosome reaction. In acrosomal spermatozoa of sea lamprey *Petromyzon marinus*, however, we found chymotrypsin-like activity instead of trypsin-like activity (Dabrowski et al. unpublished data). The lack of a micropyle in lampreys suggests the potential role of the chymotrypsin-like activity in the acrosome reaction and in egg penetration. At present, a mechanism of control of chymotrypsin-like activity is not known due to lack of data concerning proteinase inhibitors in sea lamprey semen.

Enzymes

Numerous enzymes have been detected in seminal plasma. We found esterase activity after electrophoresis (one or two bands) of teleost fish seminal plasma (Figure 2), which confirmed earlier data (Breton et al. 1974). This enzyme co-migrated during electrophoresis in the same position as the esterase from blood plasma (Ciereszko et al. unpublished data) and may be similar to an enzyme previously detected in fish blood serum and plasma (Ellis and Grisley 1985). About twenty enzymes of low activity have been identified in seminal plasma (Breton et al. 1974, De Kruger et al. 1984, Lahnsteiner et al. 1993a, 1995b, Billard et al. 1995a). Their origin and role are unclear. Likely, they originate from testes, accessory glands, and the spermatic duct, or some may originate from blood. It is unclear if these low activity enzymes are secretory products of the reproductive system and have a role in supporting or protecting spermatozoa or if they originate from apoptotic epithelial or sperm cells. In the latter case, activity of these enzymes may have a diagnostic value for describing damage to spermatozoa.

Other Peptides and Proteins

An androgen-binding protein was found in seminal plasma of rainbow trout (Foucher and Le Gac 1989), which was similar to steroid-binding proteins present in other vertebrates (Foucher et al. 1992). This androgen-binding protein is involved in the control of spermatogenesis and may participate in the acquisition of sperm motility (Miura et al. 1992). It is possible that some other proteins of seminal plasma may have anti-oxidative properties, because they can protect ascorbic acid from oxidative destruction (see above). It is not clear if these properties may be attributed to already described proteins or to unknown proteins. The presence

of several small peptides has been reported for common carp seminal plasma, but their role is unknown (Billard and Menezo 1984).

Seminal Plasma Substances Originating from Spermatozoa

At least ten proteins were recognized in seminal plasma of rainbow trout that were antigenically related to sperm proteins and co-migrated during electrophoresis with membranous or flagellar sperm proteins (Loir et al. 1990). These proteins were present in seminal plasma, sometimes in noticeable amounts, at the end of the spermiation period. This suggests that they originated from spermatozoa and were released from damaged sperm into seminal plasma. Levels of one of these proteins, with a molecular weight of 42 kDa, may be related to sperm quality and suitability of spermatozoa for cryopreservation (Maisse et al. 1988, Malejac et al. 1990). The amount of this protein may be related to sperm quality, because rainbow trout ejaculates with the best fertility after cryopreservation exhibited a lack of this protein in seminal fluid (Loir et al. 1990).

Two enzymes of sperm origin were identified in seminal plasma. Activities of these enzymes usually were low in seminal plasma and several times higher in spermatozoa. Seminal plasma enzyme concentrations may increase as a result of damage to sperm membranes. For this reason they may be used in evaluation of sperm quality. The activity of aspartate aminotransferase (AspAT) and lactate dehydrogenase (LDH) could be employed for this purpose (Schmehl et al. 1987, McNiven et al. 1992). For example, an excellent regression ($r^2 = 0.995$) between LDH activity in seminal plasma and percentage of killed spermatozoa of rainbow trout (McNiven et al. 1992). We found that AspAT activity of milt of rainbow trout and whitefish increased in seminal plasma and decreased in spermatozoa after short-term storage on ice, indicating a transfer of AspAT from spermatozoa to seminal plasma (Ciereszko and Dabrowski 1994). The possible origin of low molecular weight seminal plasma substances from spermatozoa has been discussed earlier.

Correlation between Seminal Plasma Mineral and Organic Substances and Fertilization Success

No single parameter of milt, including sperm motility, has been proven to be an unequivocal predictor of sperm fertilizing ability. However, many parameters of seminal plasma show moderate correlation with sperm fertilization rate. A positive correlation has been found between fertilization rate of Atlantic salmon *Salmo salar* spermatozoa and osmolality ($r = 0.57$), concentrations of sodium ($r = 0.60$), potassium ($r = 0.48$) and glucose ($r = 0.42$), and sodium/potassium ratio ($r = 0.52$) (Aas et al. 1991). These authors suggested the threshold value of 87 mM of sodium in seminal plasma of Atlantic salmon for an adequate fertilization rate.

A positive correlation between seminal plasma parameters and semen quality suggests that this relationship might be useful for evaluating quality of cryopreserved fish semen. For example, in a study of four parameters of seminal plasma (glucose, glycerol, sodium and potassium), concentrations of glucose correlated significantly with fertilization success of thawed semen ($r = -0.63$). A negative correlation between concentration of glucose and sperm fertilizing ability was unexpected in respect to data for fresh semen of Atlantic salmon (Aas et al. 1991). It was suggested that this correlation (and the one for glycerol) could indicate deterioration of the mineral and metabolic regulation capacity within the testes (Piironen 1987). This agrees with data demonstrating the importance of glucose metabolism during spermatogenesis (Soengas et al. 1993). On the other hand, data showing the importance of sodium and potassium agree with results for Atlantic salmon and indicate that these ions may be

related to semen quality and suitable for cryopreservation. A positive correlation (r = 0.63) was found between seminal plasma osmolality and post-thaw fertility rate of rainbow trout semen (Lahnsteiner et al. 1995a, 1996c), which agrees with data for fresh semen of Atlantic salmon (Aas et al. 1991). However, these authors found a negative correlation (r = -0.60) between seminal plasma pH and post-thaw fertility rate. In their experiment it appeared that a decline in sperm fertilizing ability occurred in milt samples with pH higher than 8.2. Other seminal plasma components affected fertilizing ability to a less, but significant extent. The relationship between fertilization rate and seminal fluid triglycerides was described by a positive linear regression. Relationships between LDH and β-D-glucuronidase activities were described by negative quadratic regressions. The nature of these regressions was not clear. High LDH activity is likely an indicator of sperm damage, whereas levels of triglycerides may reflect secretory functions of spermatic duct epithelium and β-D-glucuronidase may indicate the beginning of degeneration processes and the senescence of semen (Lahnsteiner et al. 1996c). These results suggest that further studies are necessary to elucidate which seminal plasma characteristics are related to sperm quality and which reflect a functioning of the fish reproductive system.

Contamination of Seminal Plasma

Even small amounts of contaminants can seriously affect the biochemical parameters of seminal plasma and if undetected may produce false results. The most effective milt collection method is the use of a catheter, although in practical conditions milt is usually collected by stripping. This latter method may lead to contamination of milt with water, slime, feces, blood, or urine. All of these contaminants, with urine potentially being the most serious, may lead to a decrease of sperm quality and decrease the usefulness of semen for cryopreservation. This presumption is based on the harmful nature of contaminants, but their effects on sperm cryopreservation have not been studied. It is recommended by most investigators that any contaminated milt should be discarded, however it is difficult to detect minute amounts of contamination.

Contamination of milt with slime may seriously affect biochemical parameters of seminal plasma and sperm function. Skin mucus of fish contains many potential contaminants, including proteins (Hjelmeland et al. 1983, Zilberg and Klesius 1997). However, contamination of milt with skin mucus and water can easily be avoided by practicing care during stripping.

Contamination of milt by feces may happen quite often but can be monitored visually. Even small amounts of feces produce an increase of alkaline phosphatase activity in seminal plasma (Ciereszko and Dabrowski 1994). Consequently, activity of this enzyme may be an indicator of fecal contamination.

The presence of blood in milt can be monitored visually, however synthetic carotenoids present in some commercial diets can produce a pink color in milt which may make it difficult to distinguish blood contamination (Ciereszko and Glogowski unpublished data). The concentration of protein and anti-proteinase activity in blood is greater by more than 20-fold than that of seminal plasma (Loir et al. 1990, Ciereszko et al. 1996b). Therefore, blood contamination of milt may increase amounts of these components in seminal plasma.

Contamination of stripped milt with urine seems to be the most serious problem because it is difficult to detect. This contamination can be partially decreased by clearing the bladder before milt collection using a technique described by Piironen (1994). Due to the low osmolality of urine, its addition to milt of freshwater fishes may cause partial activation of sperm that results in a decrease of ATP concentration in spermatozoa and decreased sperm motility (Percherc et al.

1995a, 1995b). It is possible that semen samples classified as being of poor quality have been activated by urine during sampling (Billard et al. 1995a). Urine contamination of milt may be monitored by a decrease in milt potassium levels and osmolality (Rana 1995b).

Biochemical Aspects of Spermatozoa Related to Cryopreservation

Membrane Lipids

Composition and arrangement of sperm membrane lipids are important factors in determining sperm cell resistance to cryopreservation stress (Mann and Lutwak-Mann 1981, Hammerstedt et al. 1990, Parks 1997). Extensive studies on sperm lipids and their relationship to sperm quality and cryopreservation have been carried out in mammals. The major lipid classes found in mammalian sperm are phospholipids, glycolipids and sterols. The lipid composition of mammalian sperm membranes differs from that of somatic cells. One characteristic feature of sperm lipid composition is the presence of long-chain polyunsaturated fatty acids, such as docosahexaenoic (22:6) and docosapentaenoic (22:5) acids, in the phospholipid fraction.

The high proportion of polyenoic acyl chains of sperm phospholipids is related to the likely occurrence of membrane lipid peroxidation. This makes the sperm membrane highly susceptible to damage, especially when semen is subjected to storage. Sperm lipids determine susceptibility of spermatozoa to cold shock and to thermotropic phase behavior of membranes. This topic has been recently reviewed by Parks (1997). Two aspects of sperm lipid composition have been linked to cold shock. These include cholesterol to phospholipid ratio and unsaturation index of phospholipid-bound acyl chains. Cholesterol to phospholipid ratio appeared to be positively correlated with cold shock resistance. Unsaturation index of phospholipid-bound acyl chains was negatively correlated with tolerance to cooling.

Fatty acid composition of different phospholipids from several fish species has been extensively studied, and significant species-to-species differences were recorded (Drokin 1993, Labbe et al. 1995). It was also reported that the cholesterol to phospholipids ratio was significantly higher in sperm of marine fishes than it was in the sperm of freshwater fishes (Drokin 1993). This difference in ratio was suggested to be related to a higher resistance to cryopreservation stresses in sperm of marine fishes.

Several differences in the phospholipid composition of the plasma membrane of spermatozoa have been described between rainbow trout and mammals (Labbe et al. 1995). In rainbow trout these authors found lower sphingomyelin content and higher levels of phosphatidylserine and phosphatidylethanolamine. Phospatidylinositol is one of the most saturated phospholipids in mammals. On the contrary, in rainbow trout these phospholipids contain high amounts of unsaturated fatty acids. It remains to be determined how these differences in lipid composition are related to differences in sperm biology and fertilization between mammals and fish.

In a separate experiment, the effects of long-term acclimation of rainbow trout to 8 °C and 18 °C, and two dietary treatments (diets supplemented either with corn or fish oil) were tested on the characteristics of sperm plasma membranes (Labbe et al. 1995). These authors found that fatty acid profiles of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine classes were affected by the fatty acid profiles of the broodstock diets. These authors concluded that sperm membranes have the ability to incorporate the fatty acids that are most available in the diet. However, this ability is limited by the need for polyunsaturated n-3 fatty acids. This indicates that lipid composition of sperm membranes may

be controlled to some extent by diet. The rearing temperature only slightly affected the sperm phospholipid fatty acids. Thus, temperature and diet manipulation caused modifications of sperm membranes that were not significant enough to produce changes in membrane fluidity.

In a similar experimental design, the effect of rainbow trout thermal acclimation on sperm cryopreservation and lipid composition of plasma membranes was tested (Labbe and Maisse 1996). Fish were acclimated to either 8 °C or 18 °C during gametogenesis, and transferred to 13 °C at the beginning of the spawning period. It was found that the fertilizing ability of cryopreserved sperm from fish reared at 18 °C was more than twice (60 to 80% fertilization) that of fish reared at 8 °C (20 to 30% fertilization) up to 42 d after the transfer. Cholesterol content of plasma membranes, expressed as cholesterol to protein and cholesterol to phospholipid ratios were lower for fish reared at 18 °C than for those reared at 8 °C. These changes were seen up to 42 d after transfer, but not longer, which corresponds to the results of the above cryopreservation trials. This finding was confirmed by a negative correlation ($r = -0.55$) between sperm fertilization rate and cholesterol to phospholipid molar ratio. These data indicate that in rainbow trout low cholesterol/phospholipid ratio correlates with cryopreservation resistance of spermatozoa. This does not agree with the general relationship established for mammals though, where sperm resistance to cooling is related to high cholesterol to phospholipid ratio. It has been noted, however, that this relationship may be characteristic only for mammals, because rooster sperm, which are tolerant to rapid cooling, have a low value of cholesterol to phospholipid ratio as well (Parks 1997).

Changes of the cholesterol content of the plasma membranes in fish transferred from 18 °C to 13 °C were interpreted by Labbe and Maisse (1996), in agreement with the concept of homeoviscous adaptation (Sinensky 1974), as an active adaptation of the membrane to preserve fluidity as temperature decreased. This interpretation is based on the fact that changes in the cholesterol to phospholipid ratio were restricted to 42 d after transfer and were not seen later. In the conclusion of their paper, Labbe and Maisse (1996) proposed that ”the response of trout spermatozoa to acclimation temperature depends both on the acclimation length and on the range of temperature used, and that in very specific conditions, sperm fertilizing ability can be significantly improved. A low membrane cholesterol level might be one parameter responsible for sperm ability to withstand cryopreservation.” Germ cells seem to be a sensitive target to environmental cues. These cues can act during gametogenesis rather than during storage of mature spermatozoa in the spermatic duct.

These results indicate that the fertilizing ability of sperm and their usefulness for cryopreservation may depend on environmental conditions. In the future this should lead to development of specific procedures for sperm and broodstock handling to improve the quality of fresh sperm and success in cryopreservation.

Effect of Cryopreservation on Sperm Composition

During the last three decades, studies on biochemical characteristics of mammalian and avian sperm have been carried out to evaluate changes in sperm cells during cryopreservation (Pace and Graham 1970, Strzezek at al. 1981a, 1981b). The central assumption in these studies is that damage to sperm cells during cryopreservation can be monitored by measuring the levels of substances, most often enzymes, before and after cryopreservation. Localization of these substances in spermatozoa should point out which structure has been damaged. The role of these substances in sperm metabolism should allow better understanding of metabolic changes in sperm caused by cryopreservation. Such information could be crucial for optimization of

particular steps in cryopreservation technology. Searching for biochemical parameters related to sperm injury seems to be particularly justified in fish sperm since, unlike in mammals, damage to sperm membranes cannot be easily monitored by light microscopy.

Attempts have been made to select constituents of spermatozoa as potential markers of sperm injury in brook trout *Salvelinus fontinalis* and rainbow trout (Schmehl et al. 1987). The experimental design was based on a comparison of seminal plasma composition before and after plunging of milt into LN_2. A number of low molecular weight substances have been shown to be released from damaged spermatozoa, including potassium and phosphorus and free amino acids. However, due to small differences between levels of these substances before and after damage, inconsistency of the results between species, and the time-consuming assay procedures, the authors did not recommend these as markers of sperm injury. Two enzymes did show promise, though. The activities of AspAT and LDH increased more than nine times after thawing, and determination of the activities of these enzymes was easy and sensitive. Therefore, these enzymes were chosen as the most applicable for evaluation of spermatozoa damage.

The activities of enzymes and the concentrations of organic compounds were evaluated in fresh and cryopreserved semen of rainbow trout (Lahnsteiner at al. 1996a). The activities of isocitrate dehydrogenase, malate dehydrogenase, L-lactate dehydrogenase, ATPase, and the concentrations of creatine phosphate and ATP were significantly lower in thawed semen than in non-frozen semen. On the other hand, the activities of adenylate kinase and pyruvate kinase and the concentrations of ADP, lactate, pyruvate, glucose and triglyceride did not change as a result of cryopreservation. The authors suggested that some sperm enzymes could have been denatured by the cryopreservation process. The effect of freezing and thawing on sperm metabolism can be monitored by selection of enzymes released from spermatozoa during this process and used as indicators of damage to sperm. For example, the decrease in activities of enzymes of the tricarboxylic cycle (isocitrate, dehydrogenase and malate dehydrogenase), together with a decrease in ATP concentration, may indicate disturbances in sperm energy production necessary for motility. This coincides with lower percentages of motile sperm and changed parameters of movement of cryopreserved sperm as compared to fresh (Lahnsteiner et al. 1996a, Ciereszko et al. 1996c). A special caution is necessary when certain sperm enzymes are selected as indicators of injury to sperm structures, because they may be denatured by cryopreservation, yielding an underestimation of their release from spermatozoa.

Some enzyme activities of fresh semen can be used to predict cryopreservation success. A significant negative correlation between post-thaw sperm fertilizing ability and activity of acid phosphatase, and a positive linear regression for activity of adenylate kinase were found in rainbow trout (Lahnsteiner et al. 1996c). These data suggest that activity of the adenylate kinase may be used as a predictor of suitability of spermatozoa for cryopreservation. However, the use of acid phosphatase for this purpose is not clear due to the negative relationship of this enzyme with sperm quality.

It has been shown that proteins are released from spermatozoa after cryopreservation. Using electrophoresis, many sperm proteins of Atlantic salmon were observed in seminal plasma after thawing without cryoprotectants (Yoo et al. 1987). This release was decreased when glycerol or DMSO was introduced to the semen. This confirms the cryoprotective role of these substances. Our recent data (Glogowski et al. unpublished data) showed that proteins released from rainbow trout spermatozoa after cryopreservation may be observed using SDS-PAGE electrophoresis (Figure 3). Within fractions released from sperm, a protein of 42 kDa dominated.

There is a possibility that this protein may be the same one observed by Maisse at al. (1988) and Malejac et al. (1990) and can be used as an indicator of suitability of semen for cryopreservation.

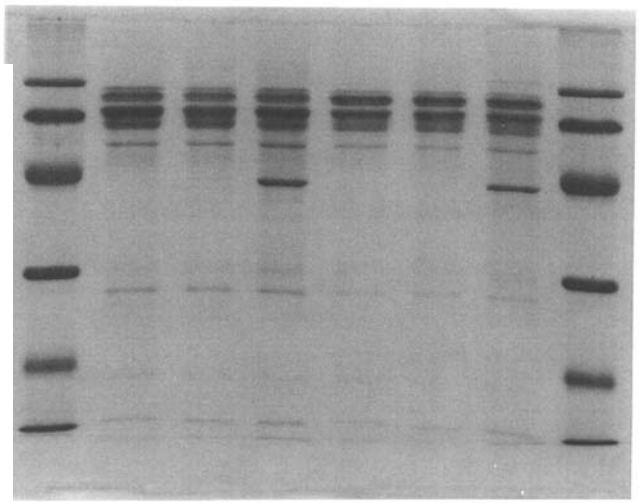

Figure 3. Loss of proteins from rainbow trout spermatozoa because of cryopreservation. Data for two individual males are presented. Lanes 1 and 8 are molecular weight markers (94, 67, 30, and 14.4 Kda). Lanes 2 and 5 are seminal plasma diluted 1:3 with extender, lanes 3 and 6 are extracellular fluid obtained after extension of milt before cryopreservation, and lanes 4 and 7 are extracellular fluid obtained after thawing. Note the presence of a 43 kDa protein in lanes 4 and 7.

Changes in enzymatic activity of sperm as a result of cryopreservation have been studied for several fish species. A decrease in AspAT activity was observed in cryopreserved sperm of rainbow trout when compared to fresh sperm (Malejac et al. 1990). The activities of AspAT and acid phosphatase in sperm of Northern pike were affected by freezing technique (straws vs. pellets) and cryoprotectant used (DMSO, glycerol, or DMA) (Glogowski et al. 1997b). Activities of both enzymes were lowest when extenders containing DMA were used. Activities of AspAT and acid phosphatase released from spermatozoa after thawing correlated negatively with fertilization rates of these spermatozoa (Figure 4). Changes in activity of both enzymes were observed in the milt of brook trout, brown trout and rainbow trout in response to cryopreservation technique (Glogowski et al. 1996). Activity of AspAT in cryopreserved spermatozoa correlated negatively with fertilization success in all three species. Similar results were found for bream *Abramis brama* (Glogowski et al. 1997a). Activity of LDH as an indicator for damage to cryopreserved rainbow trout spermatozoa has also been reported (Lahnsteiner et al. 1996b). A negative correlation ($r = -0.774$) was found between LDH liberated from spermatozoa and the percentage of motile spermatozoa. Also, the activity of LDH depended on cryopreservation procedures. Because LDH is located inside the mitochondria of rainbow trout sperm cells, its activity may be an indicator of damage to the mid-piece region of spermatozoa. Additional studies on localization of other sperm enzymes are necessary in fish to relate enzyme liberation from sperm to the damage of a particular sperm structure.

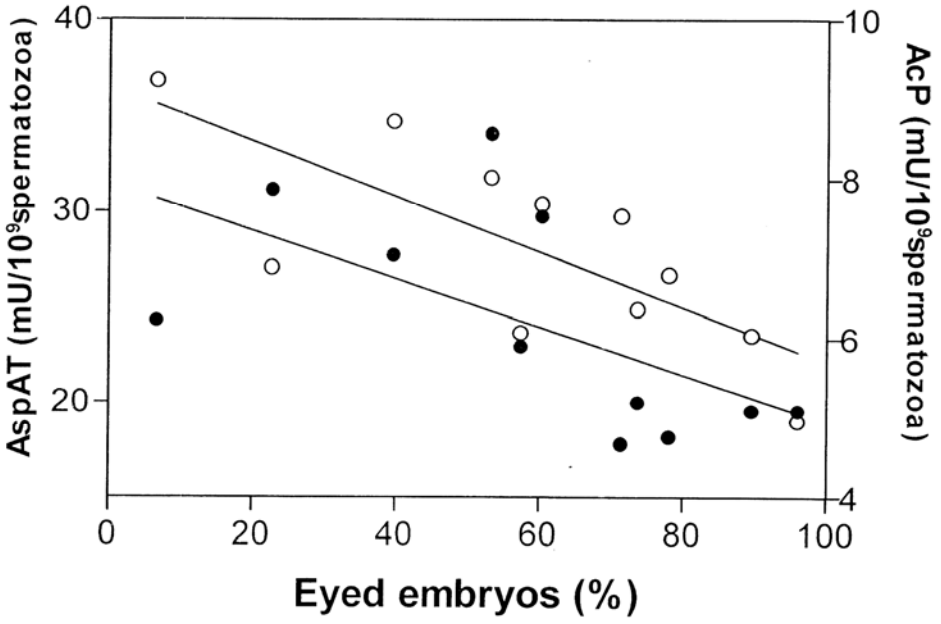

Figure 4. Correlation between sperm fertilizing ability (measured by percent eyed embryos produced) and leakage of acid phosphatase (AcP), (open points), and aspartate aminotransferase (AspAT), (closed points), from northern pike spermatozoa after thawing. Correlation coefficients were -0.76 ($P < 0.01$) for acid phosphatase and –0.59 ($P < 0.05$) for aspartate aminotransferase.

Studies on changes of sperm biochemical parameters provide new insights into the nature of the cryopreservation process. New information includes identification of substances released from sperm, their relation to sperm structure and function, and changes of metabolism of sperm cells as a result of cryopreservation. Further studies in this area are warranted. However, none of the biochemical characteristics of milt studied so far may be universally used as a practical parameter of sperm quality.

Cryopreservation of Acrosomal Spermatozoa

The acrosome is a specialized sperm organelle designed to facilitate penetration of egg envelopes by spermatozoa. The penetration involves morphological events (acrosome reaction) combined with activation of acrosome enzymes with a lytic activity toward egg envelopes. This sperm structure is absent (with few exceptions) in teleost fish spermatozoa, but is present in the acipenserids and other ancient fishes. Therefore, successful cryopreservation of acrosomal spermatozoa has to produce intact acrosomes after thawing. Recently trypsin-like activity was detected in lake sturgeon spermatozoa, which is similar to acrosin (one of the most universal acrosomal enzymes) (Ciereszko et al. 1994, 1996c). This acrosin-like enzyme appeared to be prone to denaturation by low temperatures. Its activity decreased to 10 to 20% when sperm pellets were frozen without cryoprotectant. However, freezing of semen on dry ice in the presence of DMSO and subsequent storage in liquid nitrogen resulted in successful conservation of acrosin-like activity (Ciereszko et al. 1996a, 1996b, 1996c). These results indicate that acrosin-like activity is a potential candidate for monitoring of acrosome changes after cryopreservation of lake sturgeon spermatozoa.

Conclusions

Seminal plasma creates optimal conditions for storage of semen in the reproductive system where spermatozoa are kept in a quiescent state, their functional properties (motility and genomic packaging) are protected and metabolism is supported. Numerous components of seminal plasma are involved in these functions. Mineral components such as potassium, sodium chloride, and calcium in salmonids have a principal role in protecting sperm cell immotility and later in insuring initiation of movement at the time of release. Other ions and organic substances (free amino acids and citric acid) probably have a modulating role in sperm motility. Seminal plasma sugars, lipids and glycerol support metabolism of spermatozoa. The proteins of seminal plasma are possibly involved in protecting the integrity of sperm membranes (lipoproteins) and controlling proteinases of the reproductive tract or spermatozoa (antiproteinases). Ascorbic acid is likely involved in normal spermatogenesis, sperm storage, and protection of sperm functions. Some seminal plasma components may originate from cells of the reproductive system and spermatozoa, and possibly reflect metabolism or damage to these cells. Changes in concentration of any these components may have a diagnostic value regarding the reproductive system and spermatozoa.

Studies of sperm biochemical parameters provide new insights into the nature of the cryopreservation process. Recently collected information includes identification of substances released from sperm (of small and larger molecular weights) after freezing and thawing, their relation to sperm structures and functions (for example, a decrease of sperm ATP stores), and changes of metabolism of sperm cells, including identification of enzymes which may be denatured by cryopreservation. This information is being used to modify cryopreservation techniques. For example, oxygenation of sperm suspensions is proposed to rebuild ATP stores of fresh and cryopreserved sperm.

Some components of semen composition can be controlled by external factors such as diet and temperature. This relates both to seminal plasma (ascorbic acid) and spermatozoa (lipids). Opportunity to control, to some extent, the composition of semen opens new areas of research directed toward enhancing these sperm features which are important for successful cryopreservation. Such attempts have been already successfully initiated for sperm membrane lipids.

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Summary of Recent Developments and Advances by Authors

After 2000, a few major reviews were published on this topic. Cosson (2004) summarized the current knowledge concerning ionic and osmotic factors controlling motility of fish spermatozoa. Rurangwa et al. (2004) described both sperm motility measurements and semen quality indices, and their relationship to seminal plasma characteristics. Alavi and Cosson (2006) summarized the effects of ions and osmolality on sperm motility. Using this information and published latter references I wrote a review titled "*Chemical composition of seminal plasma and its physiological relationship with sperm motility, fertilizing capacity, and cryopreservation success in fish*" (Ciereszko 2008). This review consists of three parts and was intended to update the information presented in 2000. Below I will summarize the main points of this review supplemented with results of recently published papers.

In the first part of the review (Ciereszko 2008) updated information concerning chemical and biochemical parameters of seminal plasma as semen quality indicators is provided, with special emphasis on enzymes, lipids, minerals and antioxidants. Low levels of vitamin C in seminal plasma of the rainbow trout have been found to be associated with a high percentage of abnormal embryos in the offspring (Ciereszko et al. 1999a). Moreover, uric acid has been identified in fish seminal plasma. Uric acid has a potential role in protection of fish spermatozoa against oxidative damage (Ciereszko et al. 1999b). Recently, Lahnsteiner et al. (2010) described antioxidant systems of brown trout (*Salmo trutta f. fario*) semen and confirmed the presence of high concentrations of uric acid in seminal plasma of this species.

Ingermann et al. (2001) established that salmonid seminal plasma has a low buffering capacity. The authors link this phenomenon with the facilitation of the role of sperm duct epithelium in semen pH regulation, and thus, in the sperm's acquisition motility upon exposure to water.

The second part of the review deals with proteins of seminal plasma that can potentially be used as indicators of semen quality. Compared to the data of 2000, new proteins have been identified. Mochida et al. (1999) described the presence of a sperm immobilizing factor in tilapia that is secreted in Sertoli cells and spermatic duct. This protein is able to interact with spermatozoa (Mochida et al. 2002a). Proteolytic enzymes (gelatinases) were identified in seminal plasma (Kowalski et al. 2003a). These enzymes exhibit species-specific electrophoretic profiles and belong mostly to metalloproteases and serine proteases. Ubiquitin has been found in fish semial plasma and testis (Osaki et al. 1999, Mochida et al. 2002b). Therefore it is likely that ubiquitin-mediated proteolysis is present in the fish male reproductive tract. Compared to 2000, knowledge concerning serine proteinase inhibitors has been markedly extended (Ciereszko et al. 1998). Two serpins have been identified in rainbow trout (Mak et al. 2004) and carp seminal plasma (Wojtczak et al. 2007a). These inhibitors are characterized by a suicide inhibition mechanism and may be involved in the protection of spermatozoa from proteolytic attack of proteases in seminal plasma, damaged spermatozoa or blood cells. There are at least two protease inhibitors other than serpins in seminal plasma that remain to be identified (Kowalski et al. 2003b, Wojtczak et al. 2003). Seminal plasma of percid fishes are characterized by unique profiles of serine protease inhibitors (Nynca et al. 2010a). Recently transferrin has been identified and found together with serine protease inhibitors, to be a major protein of carp seminal plasma (Wojtczak et al. 2005). Transferrin is probably involved in iron transportation to developing germ cells. Transferrin is recognized as a multi-task protein with antioxidative and antimicrobial protection activities. Polymorphism of transferrin was found to be linked to carp

sperm motility characteristics and has been recently purified and characterized (Wojtczak et al. 2007b, Dietrich et al. 2010b).

Carboxylesterases have been identified in fish seminal plasma (Ciereszko 2008). These enzymes can be potentially involved in detoxication of xenobiotics. Lahnsteiner et al. (2004) described seminal plasma proteins of molecular mass < 50 kDa capable of prolonging and stabilizing of sperm viability. Proteins of 54, 47, and the 16 kDa were identified as major proteins involved in sperm viability prolongation (Lahnsteiner 2007). However, positive effects of other unknown components of the seminal plasma on sperm viability have been suggested by this author as well. New proteins recently identified in fish seminal plasma include apolipoprotein C (Nynca et al. 2010b) and parvalbumin (Dietrich et al. 2010b). The latter is present both in seminal plasma and spermatozoa and presumably is a part of Ca^{2+}-mediated mechanisms of sperm activation in carp.

Li et al. (2009) provided a comprehensive review concerning sperm proteins in teleostean and chondrostean (sturgeon) fishes. These authors demonstrated specific effects of cryopreservation on proteins of sperm of common carp (Li et al. 2010). Eleven specific proteins related to the cryopreservation process have been identified, three as specific membrane proteins (N-ethylmaleimide-sensitive fusion protein attachment protein alpha, cofilin 2, and annexin A4) involved in membrane trafficking, organization, and cell movement; six as cytoplasmic enzymes (S-Adenosylhomocysteine hydrolase, Si:dkey-180p18.9 protein, lactate dehydrogenase B, phosphoglycerate kinase 1, transaldolase 1, and esterase D/formyl-glutathione hydrolase) involved in cell metabolism, oxidoreductase activity, and signal transduction; and two as transferrin variant C and F.

The third part of the review deals with factors affecting seminal plasma composition and semen quality. Numerous factors have been identified, including season, temperature, salinity, nutrition, antinutritional factors, stress, hormonal stimulation, and milt contamination. Seasonal changes in proteins and antiprotease activity have been described with emphasis on demonstrating their lower values at the end of the reproductive season (Ciereszko et al. 2004, Wojtczak et al. 2007c). However, such changes were not observed for seminal plasma ions (Alavi et al. 2008). The latter recorded seasonal changes for osmolality though (Alavi et al. 2010). Phase-shifted phototermal cycles produce significant changes in seminal plasma parameters (Tate and Helrfich 1998). Temperature adaptation changes have been reported for seminal plasma low molecular weight compounds of carp (Emri et al. 1998) and Arctic charr (Atse et al. 2002). Acclimation to sea water may modulate the composition of seminal plasma, including glucose concentrations (Atse et al. 2002) and osmolality (Linhart et al. 1999). It is well known that the maturation of spermatozoa is related to an increase of seminal plasma pH and fluidization of milt. Such phenomena are also observed after hormonal stimulation (Clearwater and Crim 1998). Differences in hormonal stimulation protocols can influence specific changes in seminal plasma composition (Linhart et al. 2003a). Although direct evidence is lacking, it is likely that endocrine disruptors will modify composition of seminal plasma, because their effects on sperm parameters have already been reported (Jobling et al. 2002, Gill et al. 2002).

Compared to information published in 2000 concerning the contamination of milt a description of blood cells in milt has been reported (Ciereszko et al. 2004). Linhart et al. (2003b) demonstrated the activation of tench (*Tinca tinca*) spermatozoa by urine. Poupard et al. (1998) provided further information concerning depletion of intracellular ATP stores due to urine contamination of carp semen. Dreanno et al. (1998) reported data concerning the deleterious effects of urine on sperm of marine fish. Sequential stripping of fish milt influences osmolality of

seminal plasma. In rainbow trout an increase in osmolality was observed due to a decrease in urine contamination of milt (Dietrich et al. 2005).

Data concerning nutritional effects on fish semen are very limited (Izquierdo et al. 2001). Most data concerns anioxidants (see above) and unsaturated fatty acids. These fatty acids have been shown to affect sperm volume and concentration of sea bass (Asturiano et al. 2001). Henrotte et al. (2010) demonstrated dietary effects of n-3/n-6 ratio on biochemical composition of Eurasian perch semen.

Plant-derived ingredients of fish diets contain numerous antinutritional substances, including gossypol. Gossypol is a yellow pigment indigenous to the cotton plant genus *Gossipium* with potent male contraceptive activity. Exogenous gossypol inhibits motility of yellow perch (Ciereszko and Dabrowski 2000) and sea lamprey sperm fertilizing ability (Rinchard et al. 2000). Feeding experiments conducted on diets containing cottonseed meal have resulted in a gradual increase of gossypol in the fish reproductive tract and its binding to seminal plasma proteins (Dabrowski et al. 2000, 2001). Soybean contains phytoestrogens, such as genistein and daidzein. These substances are endocrine-disrupting agents possessing estrogenic activity in vitro. Bennetau-Pelissero et al. (2001) have demonstrated that feeding rainbow trout with genistein-enriched diets accelerates testicular development and a decreases sperm motility and concentration at spawning. Feminization of males has been reported as well (Kiparissis et al. 2003, Pollack et al. 2003). Discharges from paper industry, such as wood pulp and mill effluent from bleached kraft mills are another source of phytoestrogens, especially for fish in rivers (Kiparissis et al. 2001).

Stress can significantly affect fish reproduction especially in aquaculture conditions (Schreck et al. 2001, Bayunova et al. 2002). The direct effect of stress on fish semen is related to its dilution effects. Allyn et al. (2001) have demonstrated a decrease in semen osmolality of white bass exposed to transportation stress. Such a decrease produced a premature sperm activation, an effect similar to that produced by the contamination of milt with water or urine.

Recently, Lahnstainer and Radner (2010) identified lysozyme activities and immunoglobulin concentrations in seminal plasma and spermatozoa of different teleost species. These proteins appeared to be related to sperm viability and motility.

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New Chapter

Sperm Nuclear Basic Proteins and Sperm Chromatin Organization in Fish

Juan Ausió, Núria Saperas and Manel Chiva

Overview

Sperm nuclear basic proteins (SNBPs) have been shown to consist of three main types: histone (H type), protamine-like (PL type) and protamine (P type). Fishes represent a unique group of vertebrates in that it includes species that contain representative proteins of each type. Phylogenetically, the different SNBP types exhibit a sporadic distribution that is not random and is the result of vertical evolution. Each of the SNBP types bind to DNA in a distinctive way providing the nuclei with a highly compacted chromatin organization that is characteristic of the sperm head. An improved understanding of the packing of chromatin has implications for aquaculture. The diverse nature of the SNBPs involved in fish sperm provides an excellent tool for further understanding chromatin epigenetics.

The Main SNBP Types

In the eukaryotic nucleus DNA is found associated with chromosomal proteins forming a nucleoprotein complex called chromatin (van Holde 1988). This complex enables the compaction of large eukaryotic genomes within the limited space of the nucleus. It also allows for an elaborate regulation of gene expression. Therefore, the nucleus has a highly dynamic nature (Schneider and Grosschedl 2007) where the chromatin exists in an equilibrium between different association states. In this process, individual and often extended chromatin fibers (euchromatin) serve as a template for transcription, and fibers with a different extent of association (heterochromatin) are involved in gene silencing. Thus, heterochromatin represents the most condensed state of chromatin. In a given cell there is a constitutive heterochromatin fraction that is common to different cell types such as the chromatin localized at centromeres and telomeres. Another heterochromatin fraction varies within chromosomal domains in different cell types and among chromosomes (for instance X-chromosome inactivation in mammals) and is referred to as facultative heterochromatin (Trojer and Reinberg 2007). The chromatin organization of the sperm nucleus represents an extreme situation in which almost all the chromatin is fully compacted and heterochromatinized.

The high extent of compaction of sperm chromatin is achieved by three types of structurally different chromosomal proteins generically referred to as sperm nuclear basic proteins (Ausió 1999). The three types as indicated above include the histone type (H type), the protamine type (P type) and the protamine-like type (PL type) (Ausió 1999). These types can be differentiated by electrophoretic analysis (Figure 1A). The name histone was coined by Kossel to describe the tissue (histos) and peptone nature of the extracts prepared from the nuclei of goose erythrocytes (Kossel 1884) and the proteins they represent correspond to H type SNBPs. Proteins of the H type (Figure 1A, lane 1) can be grouped into two major groups, core histones

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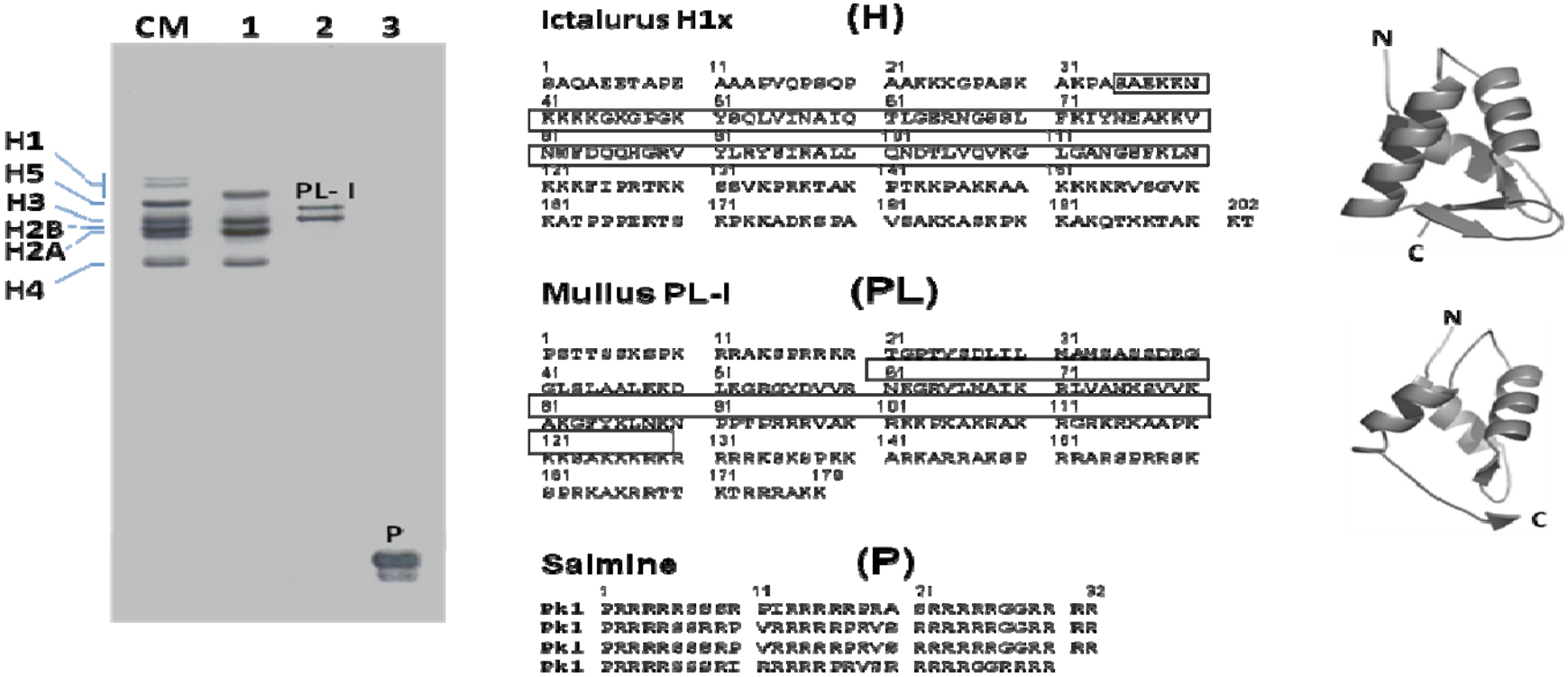

Figure 1. Structural characterization of the three main types of SNBPs in fish. (Left panel) Acetic acid (5%)-urea (2.5 M) -polyacrylamide gel electrophoresis of the SNBPs of fish species representative of each of the three main types. Lane 1: *Ictalurus punctatus* (channel catfish) (H type); lane 2: *Mullus surmuletus* (red mullet) (PL type); lane 3: *Oncorhynchus keta* (chum salmon) (P type); CM: chicken erythrocyte histones used as a marker. PL-I: protamine-like I; P: protamine. The position of the histones H1/H5, H2A, H2B, H3 and H4 is indicated in the left hand side of the gel. (Middle) Amino acid sequences representative of each of the main SNBP types: *Ictalurus* histone H1x (from *I. punctatus*, accession number: AAQ99138) (Evans et al. 2005); *Mullus* PL-I (from *M. surmuletus*, accession number: Q08GK9) (Saperas et al. 2006); protamines (salmine) from *O. keta* (Hoffmann et al. 1990). The boxes highlight the sequence corresponding to the winged helix domain (WHD) of these proteins and the numbers correspond to the amino acid location along the sequence. (Right) Tertiary structure organization of the trypsin-resistant WHD of linker histones (H1/H5) (Ramakrishnan et al. 1993, Cerf et al. 1994) (upper image) in comparison to the predicted tertiary structure for the WHD of the PL-I protein of *M. surmuletus* (Saperas et al. 2006). C and N denote the C- and N-terminal ends respectively.

and linker histones. The former includes histones H3, H4, H2A, H2B and their non-allelic variants (Ausio 2006). They have an amino acid composition that is rich in lysine and arginine (10-15 mol % of each) and their molecular masses range from 10,000 to 14,000. They contain a histone fold domain (Arents and Moudrianakis 1995) that is flanked by N- and C-terminal tail domains that exhibit a disordered conformation in solution.

The linker histones or histones of the H1 family have a lysine-rich amino acid composition (20-30 mol %) with molecular masses of around 20,000. The primary structure of these proteins often exhibits a high extent of microheterogeneity (Cole 1987). Their distinctive tertiary structure feature is the winged helix domain (WHD) (Ramakrishnan et al. 1993) (Figure 1) which is surrounded by N- and C-terminal tails. These disordered regions have been shown to adopt an important secondary structure organization upon interaction with DNA (Roque et al. 2005, Vila et al. 2001a,b).

Protamines were first described in fish. The name was used by Miescher in 1874 to refer to an "organic base" extracted from the nuclei of the sperm of salmon (Miescher 1874). Over the years these proteins have been extensively characterized (Felix 1960, Ando 1973, Oliva and Dixon 1991). The protamine type (Figure 1, left, lane 3) consists of a structurally heterogeneous group of arginine-rich proteins which are usually smaller than histones ($4{,}000 \leq M_r \leq 10{,}000$). They lack any secondary structure in solution but it is possible that like histone H1, they adopt a helix-like organization upon interacting with DNA. In addition to arginine these proteins may also contain cysteine, an amino acid that is seldom found in any other chromosomal proteins. Cysteine-containing protamines are ubiquitously present in the sperm of some chondrichthyans (Kasinsky 1989, Saperas et al. 1997, Chiva 1995, Wouters-Tyrou et al. 1998). Like histone H1, protamines can in certain instances, exhibit amino acid sequence microheterogenity (Lewis et al. 2003) (Figure 1, middle).

The Fish SNBP Types and Their Phylogenetic Distribution

The H, P and PL type SNBPs are widely distributed throughout the animal kingdom (Bloch 1976, Kasinsky 1989, Ausió 1995, Eirín-López et al. 2006) and organisms containing proteins representative of each type are found in protostomes and deuterostomes (Ausió 1999, Eirín-López et al. 2006). It has now been demonstrated that the H and PL types are structurally and phylogenetically related through linker histones (histones of the H1 family). An H – to – PL – to – P transition has been proposed to be involved in the evolution of these chromosomal sperm proteins and is now well documented. Although several reversions between types appear to have occurred in the course of evolution, SNBPs of the P-type are generally more abundant in species at the higher tips of the phylogenetic tree (Ausió 1999).

In fish, species containing proteins representative of each of the three main SNBP types are present (Saperas et al. 1994, Saperas et al. 1996, Saperas et al. 1997) (Figure 2). Species of the H-type contain histones that are compositionally almost indistinguishable from the histones that are found associated with chromatin of somatic tissues as reported in agnathans (Saperas et al. 1997) and in Cypriniformes (Munoz-Guerra et al. 1982). Nevertheless, quite often the linker histone/core histone stoichiometry is slightly higher (Saperas et al. 1993a) as would be expected from the role of linker histones in the folding of the chromatin fiber and from the silent nature of the transcriptionally inactive sperm chromatin.

Interestingly, the PL-I proteins that have been observed in fish have been found to be related to histone H1x in other vertebrates (Frehlick et al. 2007). Histone H1x is a replacement linker histone associated with chromatin that is refractory to micrococcal nuclease digestion and whose gene occupies a solitary location in the genome and gives rise to polyadenylated mRNA transcripts (Happel et al. 2005). These are characteristics that are commonly shared with PL proteins (Eirín-López et al. 2006, Saperas et al. 2006). Of note, histone H1x has been identified in the sperm of the channel catfish *Ictalurus punctatus* (Frehlick and Ausió, unpublished) which is a fish representative of the H-type (Figure 1).

Fish protamines are very variable in the number and composition of their amino acids. While most teleost protamines are higly arginine rich (50-70 mol %) (Ando 1973, Oliva and Dixon 1991, Frehlick et al. 2006) chondrichthyan protamines are compositionally more variable and often include lysine and cysteine as well as arginine (Kasinsky 1989, Saperas et al. 1997, Chiva 1995). In addition to their structural variability, fish protamines exhibit different extents of sequence microheterogeneity presumably arising from the presence of multiple copies of the protamine genes. While microheterogeneity has been well documented in different species of the orders clupeiformes (Ando 1973) and salmonifiormes (Oliva and Dixon 1991) (Figure 1) other fish species (such as the yellow perch *Perca flavescens*) (Chao and Davies 1992) contain only one or two protamine genes (Chao and Davies 1992). Although the functional implications of such protamine microheterogeneity are not clear, it has been suggested that it arises at the onset of gene duplication (Hunt et al. 1996) which is involved in the evolution of protamine genes (Oliva and Dixon 1991).

It is evident that the P type is the most extensively represented group of SNBPs in fish (Figure 2). Hence, it is not surprising that the first early characterization of protamines was in this group of vertebrates (Miescher 1874). The H- and PL- types are by far less abundant. While SNBPs of the H-, PL- and P type are all present in sperm of the external fertilizing species referred to as aquasperm in the sperm classification of Jamieson (Jamieson 1991), sperm from the internally inseminated species (introsperm) only contain SNBP of either the PL- or P type (see next section for further discussion).

As it was initially shown, the sporadic distribution of the three SNBP types (Saperas et al. 1993a) illustrated in Figure 2 is not random (Saperas et al. 1994). It can be explained by a process of vertical evolution resulting from a repeated and independent loss of expression of protamine genes (or loss of the genes themselves) that took place during the diversification of the orders of this group (Saperas et al. 1994). Indeed, with only two exceptions (Scorpaeiformes and Perciformes) the distribution of SNBP types is uniform within each order (Figure 2).

Further support for the vertical nature of SNBP evolution in fish comes from the observation that fish protamines, despite their relatively simple highly arginine-rich (as high as 70 mol %) amino acid composition (Saperas et al. 1994), provide excellent molecular phylogenetic markers that unequivocally allow one to trace the evolution of teleosts. It has been shown that the amino acid sequences of these proteins, despite their apparent compositional simplicity, can be used to obtain molecular phylogenies closely resemble those based on the morphological and anatomical characteristics of the species belonging to this group of fish (Frehlick et al. 2006). This is partly because like other reproductive proteins (Swanson and Vacquier 2002), protamines are among the most rapidly evolving of proteins (Oliva and Dixon 1991, Lewis et al. 2003) and hence can be used to distinguish between closely related species. A

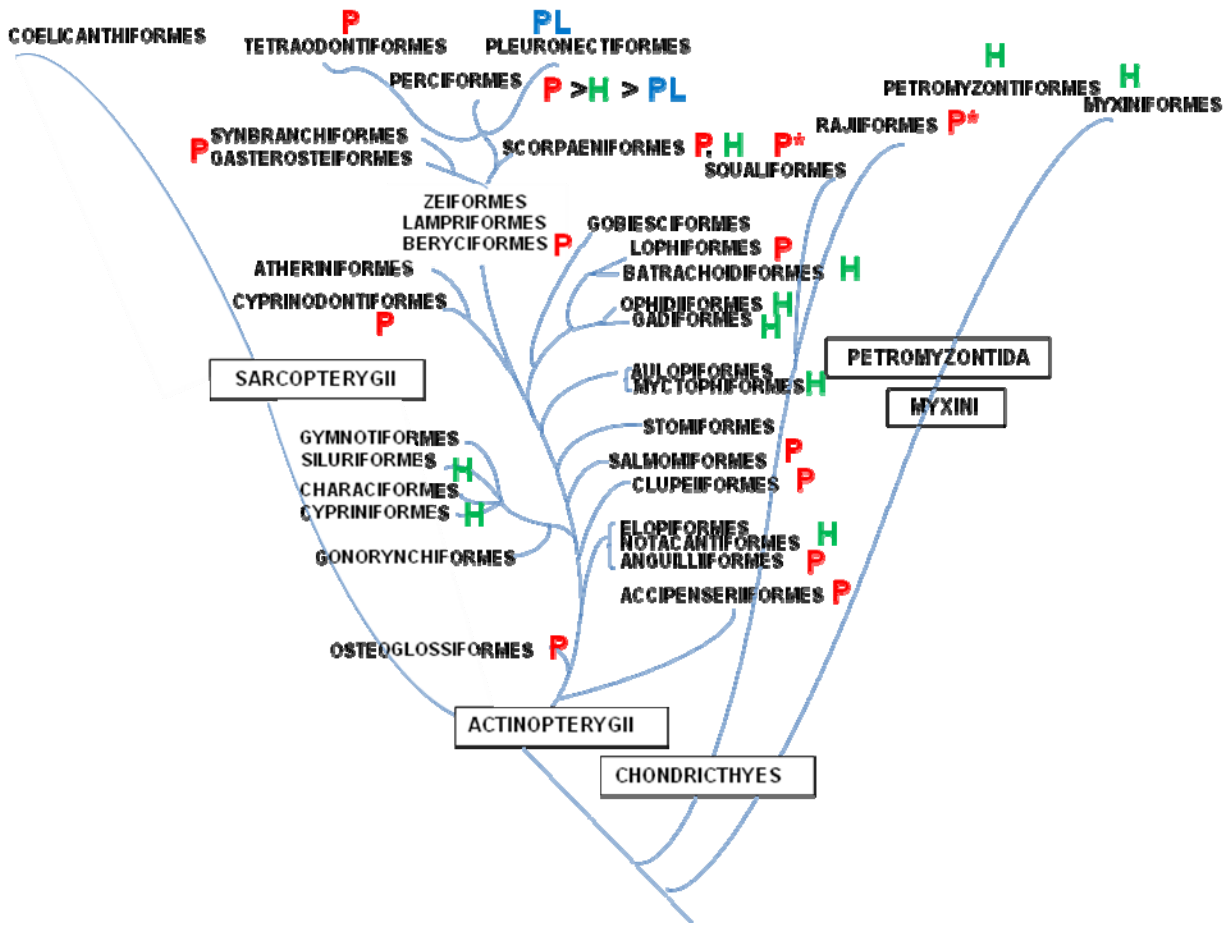

Figure 2. Distribution of the different types of SNBPs among the main fish classes and orders. The phylogenetic tree has been drawn according to Nelson (2006). The classes are highlighted by boxes. H: (H type); P: (P type) and PL: (PL type). P* designates protamines containing cysteine. For a more detailed distribution regarding individual fish species, the reader is referred to Table 2 in Saperas et al. (1994).

clear example of this can be found in the reliability with which protamines allowed the tracing of the phylogeny of closely related species of sticklebacks (Giménez-Bonafé et al. 2000).

Sperm Chromatin Organization and Nuclear Compaction

Different chromatin structures result from the interaction of the three main SNBP types with DNA (Figure 3). Histones and SNBPs of the H type organize chromatin into discrete nucleosome structures in which approximately 200 bp of DNA are wrapped about a histone core consisting of two histone H2A-H2B dimers and an H3-H4 tetramer. In the resulting repetitive nucleosomal organization, linker histones (histone H1 family) bind to the linker DNA connecting adjacent nucleosomes and in doing so they induce the folding of the nucleosome arrays into a 300-Å chromatin fiber (van Holde 1988) (Figure 3 H). This fiber can fold onto itself and produce highly condensed structures consisting of interdigitating 300 Å fibers (Daban 2003).

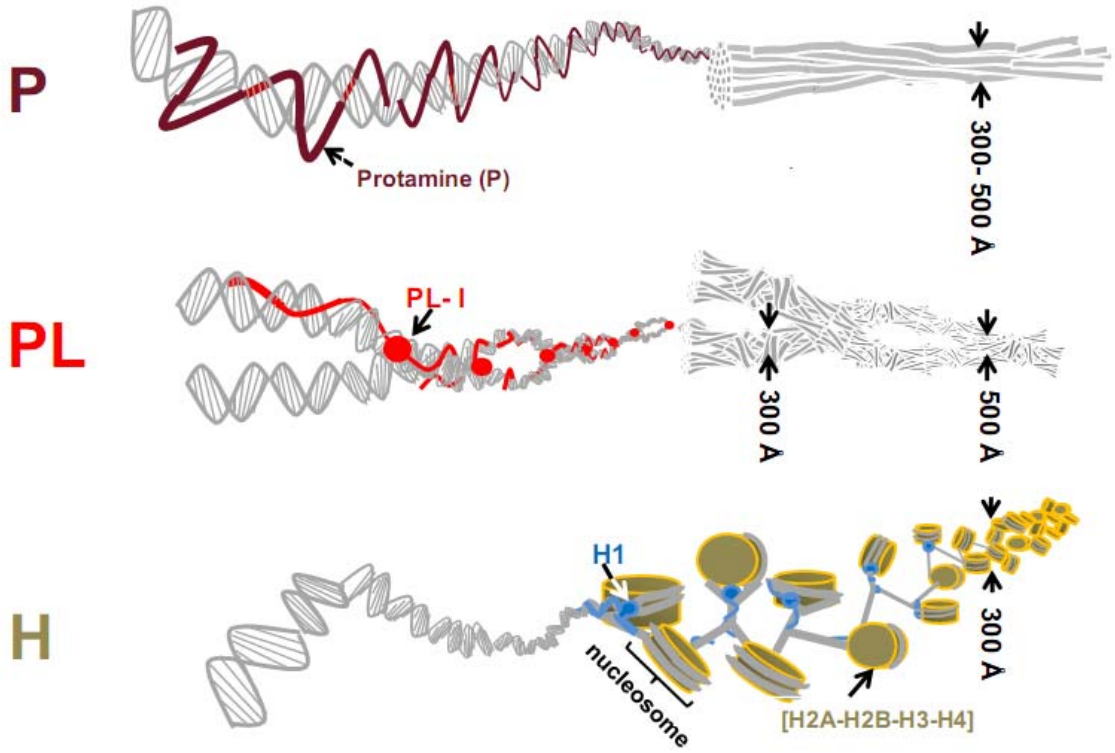

Figure 3. Chromatin organization resulting from the association of DNA (grey double helix) with the three main types of SNBPs.

Protamines bind to the major groove of DNA (Subirana 1991) resulting in nucleoprotamine complexes that are essentially neutrally charged and in which the DNA is tightly packed (Figure 3, P) (Balhorn 2007). The presence of cysteine in some protamines such as in chondrichthyans contribute to enhance the intercomplex association resulting in an extremely tight DNA organization (Balhorn et al. 1992). The first level of chromatin structure resulting from the interaction of protamine-like proteins such as fish PL-I with DNA is less clear. A hypothetical model exists (Figure 3) that takes into consideration the presence of the winged helix domain in these proteins and the ability of this domain to bind to supercoiled (intertwisting) DNA (Frehlick et al. 2007). Accordingly (as shown on the left side of Figure 3, PL), intertwined strands of DNA would be bound by PL-I proteins. The highly charged nature of the N- and C-terminal disordered ends of these proteins would neutralize the DNA charge producing nucleoprotein fibers of about 300 Å that would otherwise be similar to those produced by SNBPs of the P type. In the red mullet *M. surmuletus* PL-I accounts for >90% of the chromosomal protein component in the mature sperm (Figure 1A, lane 2) (Saperas et al. 1993b, Saperas et al. 2006). Fibro-granular structures of 250 ± 50 Å in diameter have been observed in early spermatids of this fish (Saperas et al. 1993b). Of note, it has been theoretically demonstrated that DNA condensation upon charge neutralization by interaction with SNBPs results in the formation of fibers of a relatively constant diameter of about 300 Å (Subirana 1992). This phenomenon appears to be independent of any specific protein-DNA interactions or of the structural nature of the specific SNBP involved (Casas et al. 1993).

Further compaction of chromatin beyond the 300 Å fibrogranular structures leads to the highly compacted, electron opaque, nuclear organization which is observed in the nucleus of the spermatozoa (Figure 4).

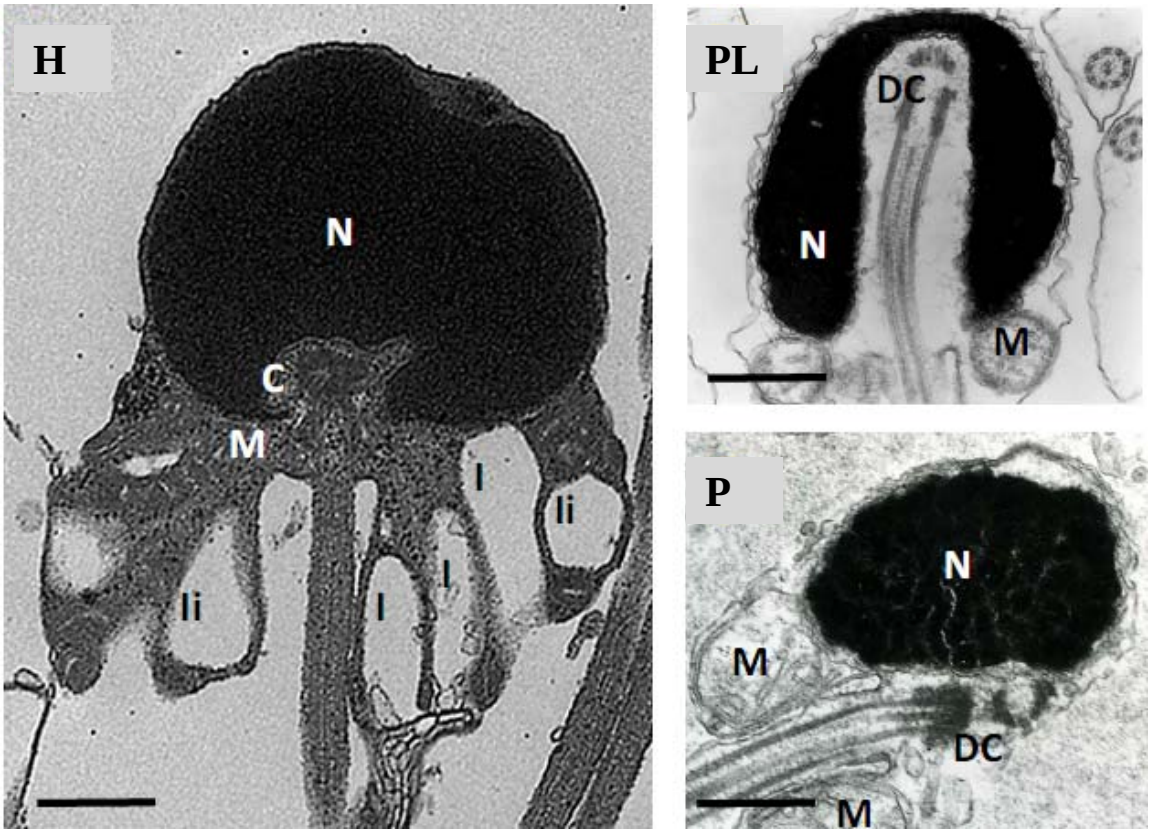

Figure. 4. Electron microscopy images of sections through the sperm heads of spermatozoa from fish species representative of each of the three main SNBP types. H, histone type, channel catfish; PL, protamine-like type, red mullet; P, protamine type, European seabass. C, centriole; DC, distal centriole; I, midpiece indentations; Ii, inclusions in midpiece indentations; M, mitochondria; N, nucleus. The bar is 0.5 µm.

The intermediate transitions that lead to this final highly condensed nuclear stage are quite diverse. For instance, in the European seabass *Dicentrarchus labrax* a representative fish of the P type, the initial 300 Å fibers are organized into coarse granules of 1500 ± 500 Å in the ripe sperm (Figure 4) (Saperas et al. 1993b). However, in the red mullet, a fish representative of the PL type, the chromatin fibers further coalesce into fibroglobular structures of 500 ± 100 Å that lead to the highly compacted nucleus of the mature sperm (Figure 4) (Saperas et al. 1993b). In this later instance the 500-Å nucleoprotein bundles appear to exhibit a highly intertwined organization (Figure 3, PL, right side) (Saperas et al. 2006).

Regardless of the details of the transitions that lead to the final compacted state of the sperm nucleus, it is the basal starting organization of the chromatin fiber at the onset of spermiogenesis that ultimately determines the extent of nuclear compaction (Ausio et al. 2007). Thus, whereas the chromatin fibers resulting from the association of the DNA with either one of the H-, PL- or P types have a similar cross-sectional diameter, the nucleosomal organization resulting from the H-type reduces the efficiency with which the DNA molecules are packed by

approximately two fold (Ausio et al. 2007) as can also be seen in Figure 4 when comparing panels P and PL with H which corresponds to the sperm nucleus of the channel catfish that contains only histones (Figure 1, left, lane 1).

In addition to packing the DNA and erasing the somatic histone epigenetic component (Ausió 1995), the chromatin-driven nuclear compaction of spermatozoa probably provides protection against external damaging agents (such as ionizing radiation, or changes in ionic strength of the media) in the journey of the sperm from the male body in search of the egg (Caron et al. 2005). In fish, this may be especially important for externally fertilizing species where the sperm (aquasperm) has to swim in either marine or fresh water environments while being exposed to potentially damaging physical and chemical agents. It is interesting to note in this regard that a significant amount of the freshwater fish contain SNBPs of the H-type. It was proposed on this basis, that this may reflect the stronger DNA binding affinity of the arginine-rich protamines and PLs (Ausió et al. 1984, Hélène and Lancelot 1982) which make the nucleoprotamine complexes more resilient to dissociation by ionic strength and hence more suitable for a marine environment (Nandi et al. 1979). Unfortunately, the H type SNBP has also been identified in marine species (Figure 2) (Saperas et al. 1994).

Despite this, it is possible that the stronger DNA binding affinity of the arginine-rich protamines compared to histones might have played a critical role in allowing the transition from external to internal fertilization (introsperm) (Kasinsky et al. 1985, Kasinsky 1989, Kasinsky 1995). While the three H, PL and P SNBP types are observed in externally fertilizing fish and other vertebrates, only the P-type is found in internally fertilizing species suggesting that the transition to internal fertilization may have imposed an important constraint to the reversibility (H→PL→ P transition) between the three SNBP types that was described at the beginning of the preceding section.

Conclusions and Future Perspectives

As described in this chapter, the SNBPs associated with chromatin in the sperm of different fish species and the chromatin complexes arising (nuclear compaction) are quite variable but fall within three well defined H-, PL- and P-types. Each of these types exhibits a characteristic chromatin folding that results in a different extent of nuclear compaction. From a practical point of view, understanding the chemical nature of the SNBPs involved in chromatin compaction of different fish species may be important for designing cryopreservation methods for the purpose of species conservation and aquaculture.

From the point of view of basic research, at a time when chromatin and its epigenetics are receiving a lot of attention, the sperm of the fish with SNBPs of the H type provide a unique system not only to address some of the questions still standing in the field of vertebrate spermiogenesis but also to better understand fundamental processes involving chromatin itself. For instance, does the wave of histone H4 acetylation that precedes replacement of histones by protamines during spermiogenesis in fish of the P type also take place in fish of the H-type? Because the chromatin in the mature sperm is fully heterochromatinized, the analysis of the histone post-translational modifications in the H-type sperm may prove to be insightful in defining the modifications associated with facultative and constitutive heterochromatin. The characterization of the linker histones involved may also be relevant in this regard.

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New Chapter

Spermatozoal Ultrastructure of Ark Clams of the Superfamily Arcacea

Wan-Xi Yang and Jun-Quan Zhu

Introduction

The bivalve superfamily Arcacea (the ark shells) includes 62 species and 18 genera in China (Xu 1997). Many species, such as *Tegillarca granosa*, *Tegillarca nodifera*, *Scapharca broughtoni*, and *Estellarca olivacea* have economic importance and commercial value. Culture of these species is developing fast in the eastern coast of China, and *in vitro* fertilization of ark clams (family arcidae) will be used widely in next decade. But in general, cryopreservation of bivalve sperm is relatively less studied than for fish species, and clams are less studied than other bivalves such as oysters.

Knowledge of bivalve sperm will assist cryopreservation in ark clams. In the past several decades, the systematic status, biogeography, and phylogenetic relationships of molluscs have been broadly studied based on morphological comparisons (e.g., Appleton and Brackenbury 1997, Galtsoff and Philpott 1960, Healy et al. 1998, Röpstorf et al. 2002). Compared to the wealth of information on gastropod spermatozoan structures, there are relatively few studies on bivalve sperm (Erkan and Sousa 2002, Daniel 1971, Franzen 1983, Grande et al. 2000, Jesperson et al. 2001, Longo et al. 1967, Popham 1979, Sousa and Oliveira 1994a,b, Sousa, et al. 1995). In previous studies of the Family Arcidae, we investigated spermatozoan ultrastructure of *Semimytilus subcrenata* (Zhu and Yang 2000) and *Tegillarca granosa* (Zhu et al. 2002). We discovered that obvious structural differences existed between these species in the acrosome and the nucleus. We also observed structures exhibiting only minor differences between species within the same genus (Zhu and Yang 2000, Zhu et al. 2002). Currently it appears that sperm ultrastructure within the superfamily Arcacea shares characteristics with the Family Arcidae. As would be expected, mature spermatozoa consisted of a head with a cone-shaped acrosome, a round nucleus and a tail region. As we have proposed previously, the morphology of acrosome and nucleus are adaptations to external fertilization (Zhu and Yang 2000, Zhu et al. 2002). The purpose of this chapter is to report ultrastructural features of four primary ark clam species (*Tegillarca granosa*, *T. nodifera, Scapharca broughtoni*, and *Estellarca olivacea*) with supplemental information on two other species (*S. subcrenata* and *Barbatia virescens*) to assist eventual cryopreservation of their sperm in the future.

Yang, W.-X. and J.-Q. Zhu. 2011. Spermatozoal Ultrastructure of Ark Clams of the Superfamily Arcacea. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 92-99.

Sample Collection and Processing

Sample Collection

Ark clams live in various habitats, such as muddy, sandy-muddy, and rocky intertidal zones, or sub-tidal zones at about 3-50 m of depth. Testis and sperm were collected for electron microscopy observations. The volume of collectable sperm was generally low, with a range of 0.5-1 mL in most species. *Scapharca broughtoni* was larger than the other species, and sperm volume was about 2 mL at each collection.

Sample Processing

Processing was based on methods described by Zhu et al. (2008). Briefly, the testes and sperm were collected, minced into pieces ~1 mm square, and placed into vials containing cold (4 ˚C) 2.5% glutaraldehyde in filtered sea water. Fixation lasted for 2 hr, and the testes were washed with filtered sea water, and post-fixed for 1 hr with 1% osmium tetroxide in filtered sea water at 4 ˚C.

The key step was to avoid osmotic shock, we used filtered sea water to replace conventional phosphate-buffered saline used for electron microscopy sample processing. Ark clam tissues have a high osmotic pressure because they live in sea water. This osmotic sensitivity was described for electron microscopy of sperm of Pacific oysters *Crassostrea gigas* (Dong et al. 2005).

After fixation, the testes were dehydrated by incubation in a series of increasing ethanol concentrations (50%, 70%, 95% and 100%), and infiltrated with mixtures (1:1, 1:3 for 1-2 h each) of propylene oxide and epoxy resin (Epon 812, Electron Microscopy Services, Inc. Hatfield, Pennsylvania, USA) before being transferred to pure resin at room temperature for 1 hr before embedding, followed by incubation for 48 hr at 60˚C. Ultrathin sections (600-900 Å, golden/gray interference color) were made using a LKB2088 microtome (Stockholm, Sweden). Sections were stained with 4% uranyl acetate at room temperature for 1 hr, counter-stained with lead citrate at (0.2%) room temperature for 4.5 min, and sections were examined with a transmission electron microscope (JEM-100CX II, Jeol, Tokyo, Japan) with an accelerating voltage at 75 kV.

Morphologic Measurement of the Acrosome and Nucleus

To characterize the acrosome and nucleus, we selected typical longitudinal sections, measured numerical values as parameters of morphological comparison, including length of the acrosome (linear length from the posterior edge to the anterior of nucleus), the width of the base of the acrosome (the width of the border between the acrosome and nucleus), the length of the nucleus (linear length from the anterior to posterior), the width of the nucleus (at the widest point).

General Morphology of Spermatozoa

The spermatozoa of ark clam are of the type I "aquasperm" (Jamieson 1991). The mature spermatozoa consists of a head beneath of a cone-shaped acrosome, a round nucleus and a tail region. Morphologic parameters and structural features among six species within the Family Arcacea are summarized in Table 1.

Morphology of the Acrosome and Subacrosomal Space

The acrosome was located at the anterior of the nucleus and was in general cone-shaped in longitudinal section. The size, height, and tip shape of the acrosome were different among four species (Figure 1): *S. broughtoni* had the tallest acrosome, *E. olivacea* had the shortest, with *T. granosa* and *T. nodifera* between. The acrosomal basal width of *S. broughtoni* was largest, and that of *T. nodifera* was smallest. The tip shape of the *E. olivacea* acrosome was the most rounded, and that of *T. granosa* was the most pointed. In longitudinal sections, the acrosomes of all four species showed a conical or inverted V-shape. The tip of the acrosomes in *T. nodifera*, *S. broughtoni* and *E. olivaceais* were flat and the tops were thin, whereas other locations along the acrosome were thicker (Figure 1). No extra cytoplasm existed between the acrosome and plasma membrane. The outer acrosomal membrane was adjacent to the plasma membrane, whereas the inner acrosomal membrane abutted dense material in the subacrosomal space.

The subacrosomal spaces in all four species were divided into two regions; electron-dense and electron-transparent. In *T. granosa*, *T. nodifera* and *S. broughtoni*, there was an axial rod in each electron-dense area, which occupied most of the subacrosomal space. In the other three species, there was also a basal plate between the base of the acrosome and the top of the nucleus.

Nuclear Morphology

Measurement of nuclei showed that there were differences in the lengths and widths among the species (Table 1). The nuclei of *E. olivacea* were the longest, while those of *T. nodifera* were the shortest. The nuclei of *S. broughtoni* were the widest, while those of *T. nodifera* were the most narrow. The nuclei of the four species, although different in breadth and length (Table 1), maintained a generally rounded appearance. The anterior and caudal poles of the nuclei tended to flatten out in the four species. The chromatin appeared condensed in all spermatozoa viewed by TEM, although some electron-lucent areas were observed in *T. nodifera* and *E. olivacea*. In the basal portion of the nuclei in all four species, there was deep V-shaped nuclear fossa, an electron-dense centriolar complex, and the initial portion of the axoneme. The anterior of the nuclei were relatively flattened and no anterior nuclear pocket was observed in the four species. The ratio of nuclear length to width of *Scapharea subcrenata* was similar to *Estellarea olivacea*, and that of *Barbatia virescens* was similar to *Tegillarea nodifera*. Among the six species, only *Barbatia virescens* had an anterior nuclear pocket. *Estellarea olivacea* had the highest ratio of acrosome-to-nucleus length, and *Barbatia virescens* ranked second.

Table 1. Comparison of morphologic parameters (mean ± SD) and structural features among six species of the superfamily Arcacea (this includes the two primary species of this chapter plus published values for two other species for comparison).

Parameter	*Tegillarea granosa* (n = 6)	*Tegillarea nodifera* (n = 8)	*Estellarea olivacea* (n = 8)	*Scapharea broughtoni* (n = 8)	*Scapharea subcrenata* (n = 6)	*Barbatia virescens* (n = 4)
Length of acrosome (μm)	0.671 ± 0.028	0.522 ± 0.020	0.410 ± 0.011	0.754 ± 0.009	0.761 ± 0.024	0.484 ± 0.017
Width of acrosome (μm)	0.878 ± 0.019	0.770 ± 0.020	0.824 ± 0.029	1.107 ± 0.017	0.965 ± 0.026	1.320 ± 0.064
Ratio of length to width of acrosome	0.766 ± 0.033	0.677 ± 0.016	0.500 ± 0.015	0.682 ± 0.011	0.792 ± 0.037	0.368 ± 0.009
Area (length by width) of acrosome	0.589 ± 0.029	0.404 ± 0.025	0.339 ± 0.018	0.834 ± 0.018	0.734 ± 0.030	0.642 ± 0.051
Length of nucleus (μm)	1.429 ± 0.056	1.459 ± 0.027	1.682 ± 0.035	1.544 ± 0.027	1.483 ± 0.029	1.571 ± 0.013
Width of nucleus (μm)	1.492 ± 0.066	1.538 ± 0.016	1.725 ± 0.026	1.740 ± 0.044	1.490 ± 0.033	1.600 ± 0.027
Ratio of length to width of nucleus	0.960 ± 0.020	0.949 ± 0.016	0.975 ± 0.014	0.890 ± 0.019	0.997 ± 0.020	0.949 ± 0.018
Area (length by width) of nucleus	2.149 ± 0.178	2.245 ± 0.055	2.906 ± 0.097	2.690 ± 0.102	2.130 ± 0.080	2.426 ± 0.046
Ratio of acrosome to nucleus length	2.155 ± 0.142	2.822 ± 0.114	4.123 ± 0.136	2.049 ± 0.036	1.957 ± 0.062	3.142 ± 0.107
Striation in acrosome	no	no	no	no	no	yes
Anterior nuclear pocket	no	no	no	no	no	yes
Number of mitochondria	5	5 (4)	5 (4)	5	5 (4)	5 (6)
Source	This study	This study	This study	This study	Zhu and Yang (2004)	Zhu and Yang (2004)

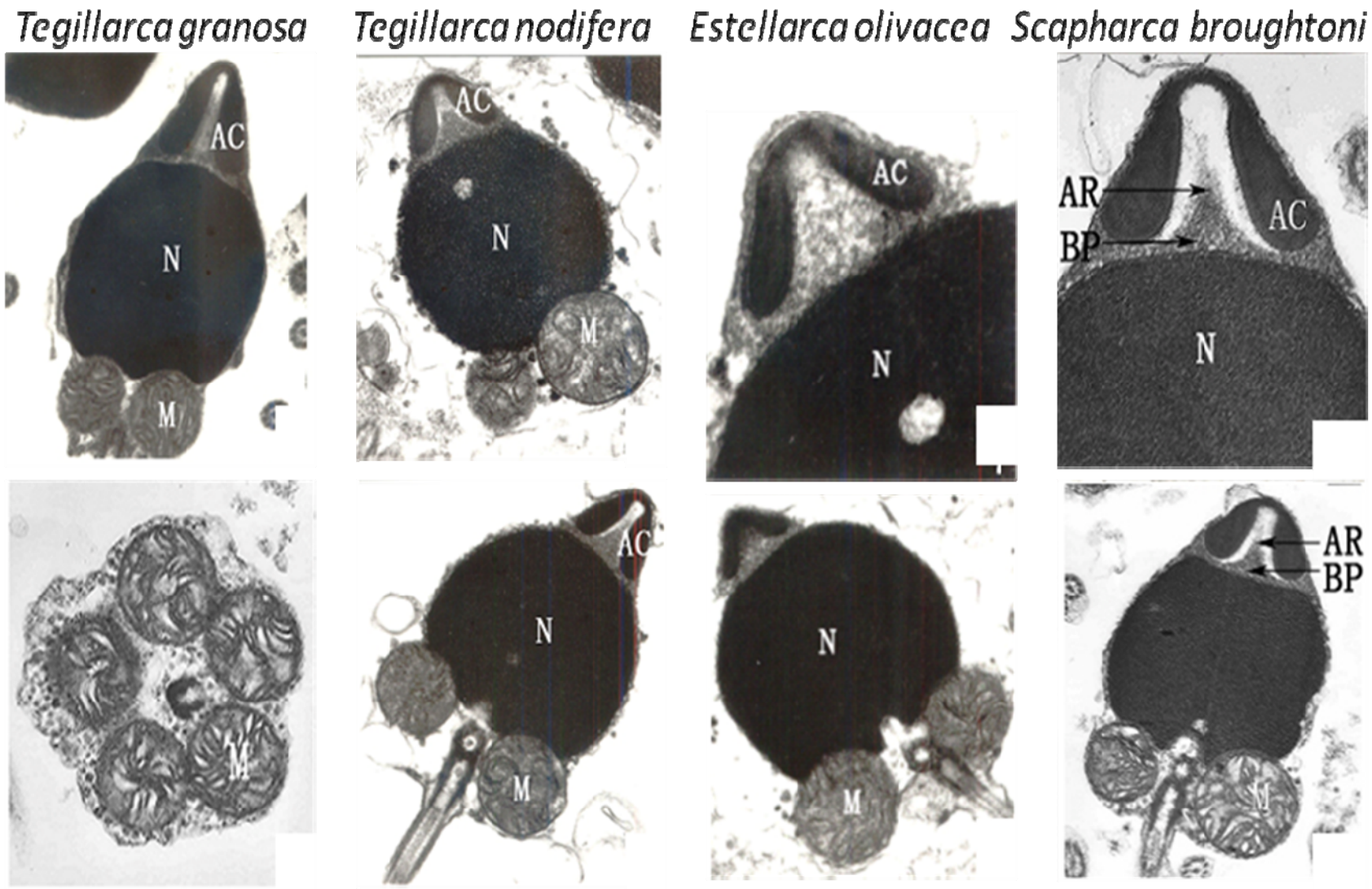

Figure 1. Sperm head ultrastructure of four ark clam species. AC: acrosome; AR: acrosomal rod; BP: basal plate; M: mitochondria; N: nucleus.

Midpiece Morphology

All four species shared midpiece features. The proximal centriole was located at the base of the posterior nuclear fossa, while the distal centriole was typically surrounded by five mitochondria. Sometimes four motochiondria were observed in *T. nodifera*, *S. broughtoni,* and *E. olivacea* (data not shown). The base of the distal centriole was closely attached to the nuclear membrane. Except for mitochondria, only few cytoplasmic inclusions existed in the short middle piece. In longitudinal sections, only two of the mitochondria could be observed, while in cross section, all five mitochondria could clearly be observed.

Endpiece Morphology

The endpiece of the spermatozoon, which was slim and whip-like was composed of the axoneme and plasma membrane. The axoneme had the typical 9+2 structure (data not shown). No other cytoplasmic inclusions were observed in the end piece except the anoneme itself.

Conclusions

Mature spermatozoa of the four species shared similar morphology, contained a cone-shaped acrosome, a round nucleus, a short midpiece typically with five mitochondria, and a tail region. In the subacrosomal space, there was a small amount of cytoplasm in the form of an axial rod and a basal plate. The chromatins were compacted, and the nucleus was solid with an inverted, shallow V-shaped posterior invagination and lacked an anterior invagination. The midpiece was short, with five spherical mitochondria surrounding the distal centriole. The axoneme had the typical 9+2 structure. Our conclusion is that the spermatozoa were of Type I "aquasperm", and the morphology of the acrosome and nucleus are adaptations to external fertilization. These structures are similar to those in other bivalves for which sperm cryopreservation has been described (e.g. Dong et al. 2005, Tiersch and Mazik 2000).

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New Chapter

Physiological Modifications to be Considered for Amphibian Sperm Cryopreservation

Darío Krapf, Emma D. O'Brien and Silvia E Arranz

Introduction

Amphibians include over 4,500 species within three major lineages: caecilians, salamanders, and anurans. These lineages are linked by several unique physiological traits. The most prominent being the lifestyle of many amphibians: the aquatic gill-breathing larval stage and the aquatic or terrestrial lung or skin-breathing adult stage.

It is now generally accepted that there has been a major population decline of amphibians in many parts of the world over the last 25 yr. The decline has been particularly evident in Australia and parts of Latin America. Although the causes of this decline are still poorly understood, it has been generally ascribed to pollution, increased UV-B radiation associated with thinning of the ozone layer and to a pathogenic chytrid fungus implicated in the decline of many species (Frías-Alvarez et al. 2008).

In the face of the inevitable further loss of species and populations, the use of amphibian sperm cryopreservation arises as potentially valuable approach, not as an economic resource, but for the conservation of amphibian biological and genetic diversity. Moreover, this approach would significantly reduce the number of males in captive breeding programs. Relatively few studies have investigated the effects of freezing and thawing of anuran sperm (Browne et al. 1998, Browne et al. 2002d, Browne et al. 2002a, Browne et al. 2002b, Browne et al. 2002c, Constanzo et al. 1998, Sargent and Mohun 2005, Fitzsimmons et al. 2007, Mansour et al. 2009). Despite the low number of studies, the general feasibility of cryopreserving amphibian sperm with successful recovery of motility capacity and fertility is evident.

Different strategies have been used to assess successful recovery after cryopreseration of amphibian sperm. Among them, sperm integrity and recovery of motility were widely used. However, due to the current status of our knowledge, this could lead to misinterpretation of actual sperm ability to fertilize an egg. *In-vitro* fertilizations with cryopreserved anuran sperm have been, in every case, far from similar to control samples using fresh sperm. In the light of these observations, this chapter will not revise amphibian sperm cryopreservation protocols, but will try to point out physiological modifications that, if properly considered in cryopreservation techniques, could result in a significant improvement of recovery rates.

Modifications Related to Extracellular Osmolarity Variation

Amphibian Ringer solution has been widely used for preparation and handling of anuran sperm suspensions (Cabada 1975b, Wolf and Hedrick 1971). Isotonicity of this solution maintains sperm viability (Cabada 1975a). This solution among others, such as Leibovitz L-15 medium, arose as a foundation for addition and testing of different protocols for cryopreservation (Sargent and Mohun 2005). In addition, sperm flagellum hyper-activation is inhibited in isotonic media. Exposure of sperm to hypotonic media triggers flagellar activation. This event is correlated with the activation of cAMP-dependent protein kinases (E. O'Brien, Institute of

Krapf, D., E. D. O'Brien and S. E. Arranz. 2011. Physiological Modifications to be Considered in Amphibian Sperm Cryopreservation. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 100-106.

Molecular and Cell Biology of Rosario, Argentina, unpublished data). Consequently, sperm maintenance in isotonic medium would prevent energy waste prior to cryopreservation. Isotonic solutions also prevent acrosome breakdown that rapidly takes place after exposure to hypotonic media (Martínez and Cabada 1996). Highly diluted solutions also produce a rapid motility decrease in spermatozoa of *Xenopus* (Inoda and Morisawa 1987), newt (Hardy and Dent 1986), and freshwater fishes (Morisawa et al. 1983b) probably as a result of sperm structure changes. In this regard, although hypotonicity promotes flagellar activation, osmotic damage to the cellular structures may result in a limitation for the duration of sperm motility and acrosome integrity.

Effect of Egg Jelly Components on Sperm Physiology

Among the different techniques employed for sperm preparation, mincing of the testes in a solution containing egg jelly components has been evaluated (Tian et al. 1997). The use of this solution is related to the requirement of the jelly coat for amphibian fertilization, as reported more than 50 yr ago (Kambara 1953). It is now known that components of the egg jelly of the toad *Bufo arenarum* induce capacitation-like changes in homologous spermatozoa (Figure 1) (Krapf et al. 2007, Krapf et al. 2009), in analogy with the concept developed in mammals (Yanagimachi 1994). However, long exposure to

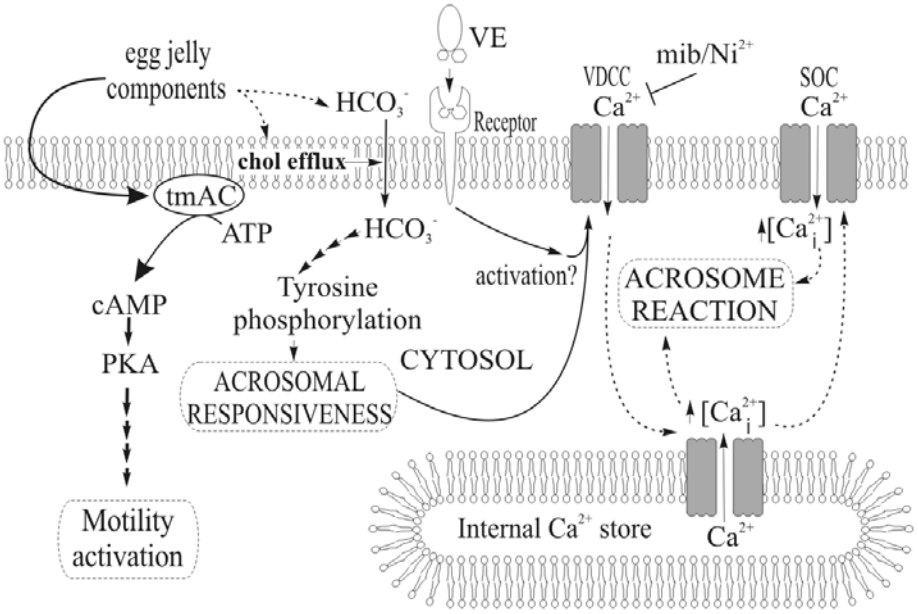

Figure 1. Working model of cellular changes elicited in *Bufo arenarum* sperm capacitation and subsequent triggering of the acrosome reaction. See text for details. Abbreviations: chol, cholesterol; SOC, store-operated Ca^{2+} channel; VDCC, voltage-dependent Ca^{2+} channel; mib, mibefradil; tmAC, transmembrane adenylyl cyclase; PKA, protein kinase A; ATP, adenosine triphosphate.

this solution renders sperm without fertilizing capacity (Fontdevila et al. 1991, Krapf et al. 2007), due to a loss of acrosomal responsiveness to acrosome reaction agonists (Krapf et al. 2009). The cascade of events leading to acquisition of fertilizing capacity of the toad sperm, promoted by incubation in diffusible egg jelly components, is acquired through an increase of protein tyrosine phosphorylation and a decrease of sperm cholesterol content (Krapf et al. 2007), even when sperm are incubated in isotonic solutions. As stated above, the onset of

phosphorylation would result in a reduction of available ATP. Moreover, cholesterol loss produces a more labile sperm due to fluidization of the plasma membrane, with reduced tolerance to temperature variations.

Changes of Extracellular Ionic Environment

Spermatozoa are extremely sensitive to alterations in the environment. As soon as sperm are released from the body, orchestrated events begin, so as to prepare for the opportunity to fertilize an egg that these cells are endowed with. Because the aim of cryopreservation is to extend the interval between sperm release and fertilization, every effort in minimizing the triggering of these events would be valuable.

Testicular plasma of frogs (Inoda and Morisawa 1987) and toads (E. O'Brien, unpublished data) are of similar osmolarity to that of amphibian Ringer medium (250 mOsmol/kg). However, the ionic composition of these media is not similar. A major difference arises from K^+ concentration: Ringer medium contains 2 mM of this ion, while testicular plasma of *Xenopus* contains ~ 70 mM (Inoda and Morisawa 1987), and that of *Bufo* contains ~40 mM (E. O'Brien, unpublished data). Although the overall osmolarity is not affected, as testicular plasma and Ringer medium each approximate 250 mOsmol, we have observed a membrane hyper-polarization of about 30 mV promoted by the sudden extracellular lowering of K^+ that sperm encounter when they are released from the testes. Moreover, when incubated in testicular plasma, sperm remain immotile. In this regard avoiding replacement of electrolytes with sugars (Browne et al. 2002d) could avoid physiological alterations that reduce post-thaw recovery.

Motility regulation pathways are not the same across aquatic species. In salmonids, sperm motility is initiated by a decrease in K^+ concentration, regardless of the extracellular osmolality (Morisawa et al. 1983a). In contrast, osmolarity seems to regulate activation of cyprinid fish sperm, where hyposmotic solutions relative to seminal plasma promote flagellar motility (Morisawa et al. 1983b). In the freshwater teleost Nile tilapia *Oreochromis niloticus*, a seminal plasma glycoprotein was shown to possess sperm immobilizing activity. It is not known if similar factors are present in seminal fluids from other freshwater species.

Role of Calcium and Bicarbonate in Sperm Physiology

Calcium and bicarbonate are each key regulators of sperm capacitation, acrosome reaction, and flagellar motility in mammals. Despite the biological relevance, the molecular mechanisms underlying these processes are still poorly understood. It has been suggested that all three events depend on the intracellular rise of cAMP induced by bicarbonate ions (Visconti et al. 1995, Okamura et al. 1985). More recently, it has been shown that bicarbonate ions directly stimulate soluble adenylyl cyclase in a pH-independent manner (Chen et al. 2000).

The presence of HCO_3^- is required for *in vitro* capacitation of mouse sperm (Visconti et al. 1995) and for acceleration of the flagellar beat (Wennemuth et al. 2003). In amphibians, HCO_3^- was recently shown to promote tyrosine phosphorylation of sperm proteins leading to the acquisition of a transient fertilizing capacity (Krapf et al. 2007). Therefore, the presence of this ion in amphibian sperm solutions could promote a capacitated state that is acquired only once in the sperm lifetime. Consequently, post-thaw fertilizing capability of sperm pre-exposed to HCO_3^- would be diminished.

Extracellular calcium is necessary for acrosome reaction in all species studied (Ishihara et al. 1984, Darszon et al. 2006, Witte and Schäfer-Somi 2007). Calcium also controls the swimming behavior of sperm and of other motile cells that use the axonemal engine (Carlson et al. 2003). Decades of study have not revealed how Ca^{2+} targets the axonemal components to alter ciliary and flagellar waveforms and thus produce responses to external stimuli (Smith 2002). In common carp *Cyprinus carpio*, membrane hyperpolarization leads to Ca^{2+} influx and initiation of sperm motility (Krasznai et al. 2000). In mammals, external Ca^{2+} seems to bind an unidentified extracellular protein that is required for HCO_3^- to engage cAMP-mediated activation of motility (Carlson et al. 2007).

Few reports exist on the requirements for calcium and bicarbonate in amphibian sperm motility. In the urodele *Cynops pyrrhogaster* activation of Ca^{2+} channels were necessary to the initiation of flagellar activation (Watanabe et al. 2003). In fishes, as in anuran amphibians, osmolarity is the main factor promoting sperm motility (Cosson 2004) but the requirements for calcium and bicarbonate have not yet been determined. Although sperm motility almost seems to be activated in a species-specific manner, all described mechanisms involve the activation of a calcium channel, leading to a Ca^{2+} influx and the initiation of sperm motility. Nowadays, it is clear that extracellular bicarbonate and calcium are both associated with sperm motility and should be taken into account to improve fertilization rates. Moreover, voltage-dependent Ca^{2+} channels have been shown to be involved in the intracellular Ca^{2+} rise observed during acrosome reaction in the Amphibian *Bufo arenarum* (Figure 1)(Krapf et al. 2009). It is still unclear whether same or different Ca^{2+} waves control sperm motility and acrosome reaction of vertebrate species.

Physiological Changes After Spermiation

The working model for the control of *Bufo arenarum* sperm physiology during fertilization is outlined in Figure 1 above. This model includes modulation of sperm responsiveness to physiological stimuli (vitelline envelope, VE) that takes place during acquisition of fertilizing capacity upon contact with egg jelly components. A voltage-dependent Ca^{2+} channel acts as a key element of the VE signal transduction. The activity of this channel could be subject to regulation during acquisition of fertilizing capacity (i.e., cholesterol depletion and tyrosine phosphorylation promoted by egg jelly components as bicarbonate). Once in an active form, the opening of this calcium channel promotes depletion of intracellular Ca^{2+} stores that precedes acrosomal exocytosis. Because sperm capacity to respond to the VE is present only for a short time, exposure to jelly components should only be restricted to *in vitro* fertilizing steps. Moreover, hyposmotic shock caused by fertilization media triggers activation of sperm transmembrane adenylyl cyclases (tmAC), increasing intracellular concentrations of cAMP. This increase in turn activates protein kinase which modulates sperm flagellar beating, in a process that requires extracellular calcium (O'Brien et al., unpublished results). It is still unclear whether independent Ca^{2+} waves govern flagellar beating and the acrosome reaction. Understanding how egg jelly components induce signaling events, as well as the function of the jelly components in supporting fertilizing capacity of amphibian sperm warrants further investigation. This knowledge would be valuable in improving cryopreservation techniques.

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New Chapter

Evaluation of Extenders for Refrigerated Storage of Koi Carp and Goldfish Sperm

Donald W. Glenn III, R. Paul Lang and Terrence R. Tiersch

Problems and Approaches to Gamete Storage

Refrigerated storage of sperm can be an effective tool to improve genetic research and lengthen the time between collection of sperm and use for fertilization of eggs. Extenders, usually consisting of a salt solution with added organic compounds, are used to dilute and preserve sperm. By adjusting the osmolality of an extender, sperm can be held immotile, and activation of eggs can be delayed (e.g., Glenn and Tiersch 2002) lengthening their useable lifetime. The extent of extender dilution can alter sperm concentration and thus affect fertilization. An activating solution (an extender with low osmolality) has been occasionally used with sperm and eggs of freshwater fish to lessen the degree of osmotic shock that can occur when solutions of different osmolality are mixed (Cognie et al. 1989, Drokin et al. 1994). The pH of an extender can also affect motility and fertilization (Roubaud et al. 1984). Extenders are occasionally used with cryoprotectants for additional protection during cold storage, and various osmolalities, pH, and dilutions have been used with different degrees of success. Studies of refrigerated and cryopreserved sperm of common carp *Cyprinus carpio* have dealt with survival and motility (e.g., Sneed and Clemens 1956, Withler 1980). Many of the reports addressing extenders and refrigerated storage in carp and other species are hard to find, inconsistent in reporting of results and conclusions (Rana 1995), and presented in several languages (see References). In addition, experimental methods are often dissimilar and difficult to repeat. This chapter is intended to show some of the problems and opportunities encountered when choosing an extender for refrigerated storage and cryopreservation. We chose to illustrate the process with a literature review and experimental work addressing common carp and goldfish.

Common carp have been selectively bred for more than 200 yr to produce domesticated ornamental varieties called koi carp. Superior koi possess distinctive coloration and markings, and individuals can be valued at greater than US$50,000. Traits of koi depend on environment, bloodline, and type of feed. Goldfish *Carassius auratus* are hardy, fast growing, easily maintained, and spawn readily in captivity. Millions of goldfish are bred each yr for sale as ornamental fish and baitfish. Improvement of procedures for selective breeding of koi and goldfish could increase the quality and value of these fish and expand our understanding of the heredity of colors and patterns. The demand for breeding husbandry of koi and goldfish will increase as the demand for ornamental ponds increases in private homes and businesses,

Like many other species of fish, koi and goldfish can be bred naturally in ponds or artificially through the use of fresh, refrigerated or cryopreserved sperm. Natural propagation of carp (sometimes stimulated by hormonal injections) has proved to be economical and efficient for commercial and research purposes. However, artificial spawning can increase the number of fry and provide greater control of specific broodstock crosses, and thus can be more economical and efficient than natural spawning. Artificial spawning using stored sperm can reduce the number of male broodstock required, thereby maximizing space in the hatchery. Sperm from one male can fertilize eggs from several females over time (even after the death of a valuable male).

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Fertilization capacity of common carp eggs with refrigerated sperm has been found to equal that obtained with fresh sperm (Hulata and Rothbard 1979), and sperm can be collected off-site and transported to the laboratory or hatchery for artificial propagation.

Study of artificial breeding with refrigerated sperm requires examination of how extenders and sperm interrelate. The storage of sperm can be increased by dilution in extenders which are used to supply ionic and osmotic conditions appropriate to prevent activation during storage and handling. Osmolality and dilutions interact with extenders to affect motility and storage. The effects of potassium and osmolality on motility has been studied in sperm of freshwater cyprinids including goldfish (Morisawa et al. 1983). Frog Ringer's solution was used to store common carp sperm for 30 d at 3-5°C (Sneed and Clemens 1956), and sperm from common carp, crucian carp *Carassius carassius*, and dace *Tribolodon hakonesis* were held in 300 mOsmol/Kg NaCl (Morisawa et al. 1983). Sperm of common carp was stored at 4°C for 12 d in extenders containing various amounts of KCl, NaCl, glucose, and DMSO (Chen et al. 1992).

The present study evaluated Hanks' balanced salt solution (HBSS), calcium-free (C-F) HBSS (Tiersch et al. 1997), and solutions of NaCl and $NaHCO_3$ for refrigerated storage of goldfish and koi sperm. Hanks' balanced salt solution has been used for storage of sperm of marine fishes such as *Cynoscion nebulosus* (Wayman et al. 1996) and *Pogonias cromis* (Wayman et al. 1997), and freshwater fish such as channel catfish *Ictalurus punctatus* (Christensen and Tiersch 1996). We sought to test the effect of dilution rates and osmolality changes with these extenders on motility and fertilizing ability of refrigerated sperm. This chapter begins with a literature review of extenders tested for refrigerated storage of common carp to illustrate the wide range of protocols and formulations available, and then provides the experiments used to test a panel of extenders. The research objectives were to evaluate the: 1) relationship among osmolality of body fluids, extender and sperm activation; 2) effect of extender osmolality on refrigerated storage; 3) effect of sperm dilution on refrigerated storage, and 4) fertilization with stored sperm.

A Review of Extenders and Sperm Refrigeration in Common Carp

Extenders have been used in most studies with sperm storage (Table 1). Undiluted sperm often quickly loses fertilizing ability due to contamination with urine (Tiersch et. al. 1997), lack of oxygen and nutrients for storage, and bacterial contamination (Belova 1982, Billard et al. 1995). Ribonucleic acid levels decreased in undiluted sperm over 24 hr when stored at 4-6 ^{o}C, decreasing fertilization (Nedovesova 1983). Suspension in extenders can reduce these problems, although excessive dilution can itself reduce quality (Paniagua-Chavez et al. 1998). Optimum osmolality and dilution must be studied for each extender, and several have produced high motility, fertilization and hatch (summarized in Appendix, end of chapter). Some extenders used for common carp are frog Ringer's solution (FR), phosphate buffer (PB), Alsever's solution (A), Cortland's fluid (C), and modified Cortland's fluid (mC). Motility was sometimes prolonged with addition of antibiotics and supplemental oxygen (Saad et al. 1988). Stirring was not recommended due to sperm fragility, and storage at lower concentrations was suggested (Belova 1981). Samples were stored at 0 to 33 ^{o}C (Musselius 1951, Bhowmick and Bagchi 1971), most commonly at ~4 ^{o}C, over a range of 3.5 to 720 h. Motility and fertilization were not different from fresh controls (Hulata and Rothbard 1979, Jahnichen 1981, Rothbard et al. 1996). Overall, refrigerated storage requires optimization of osmolality, dilution rate, container type, and temperature to maintain fertilization rates comparable to control samples.

Table 1. Extenders used for refrigerated storage of sperm from common carp *Cyprinus carpio*.

Ingredients (g/L)	Frog Ringer's solution	Phosphate buffer	Not specified	Not specified	Alsever's solution	Cortland's fluid	Diluent for freezing bull's semen	Milk-yolk diluent
NaCl	6.50				4.00	7.25		
KCl	0.14					0.38		
$CaCl_2$	0.12							
$CaCl_2 \cdot 2H_2O$						0.23		
$C_6H_5O_7Na_3 \cdot 2H_2O$					8.00		720.00	
$NaHCO_3$	0.20					1.00		
NaH_2PO_4	0.01			15.00				
$NaH_2PO_4 \cdot H_2O$						0.41		
$Na_2HPO_4 \cdot 12H_2O$		20.00						
$Na_3C_6H_5O \cdot 2H_2O$			30.00					
KH_2PO_4		2.00						
$MgSO_4 \cdot 7H_2O$						0.23		
$C_6H_{12}O_6$	2.00							
yolk				200.00			200.00	100.00
milk								900.00
fructose							12.50	
glucose					20.50	1.00		
streptomycin							1.00	
penicillin							1.00×10^6 IU	
pH		7.40	7.60					
Reference	Sneed and Clemens 1956	Sneed and Clemens 1956	Sneed and Clemens 1956	Kossmann 1973	Moczarski 1973	Moczarski 1973	Moczarski 1973	Moczarski 1973

Table 1. continued.

Ingredients (g/L)	Modified Cortland's fluid	Ringer's fluid	Not specified	Rinsing solution	Saline solution	Salt	Na-citrate	Not specified
NaCl	1.88	6.00		4.00	7.31	2-100.00		
KCl	7.20							14.91
$CaCl_2$	0.23				0.01			
$NaHCO_3$	1.00							
$C_6H_5O_7Na_3$							2-100.00	
$NaH_2PO_4 \cdot H_2O$	0.41							
$MgSO_4 \cdot 7H_2O$	0.23							
ethanol			10-20.00					
urea				3.00				
glucose	1.00							
streptomycin					50.00			
bipenicillin					$5.00x10^4$ IU			
Tris HCl								4.73
Tris					2.42			
pH					8.00			8.00
Osmolality								380
Reference	Moczarski 1973	Moczarski 1973	Moczarski 1973	Hulata and Rothbard 1979	Saad et al. 1988	Magyary et al. 1991	Magyary et al. 1991	Redondu-Muller et al. 1991

Table 1. continued.

Ingredients (g/L)	D-15	D-16	D-17	D-19	D-20	D-21	Calcium-free Hanks' balanced salt solution
NaCl	8.00	10.00	9.00	9.00	8.00	8.00	8.00
KCl	0.50	0.50	0.50	1.00	1.00	2.00	0.40
$NaHCO_3$							0.35
Na_2HPO_4							0.06
KH_2PO_4							0.06
$MgSO_4 \cdot 7H_2O$							0.20
glucose	15.00	15.00	15.00	15.00	15.00	15.00	1.00
Osmolality							305
Reference	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Chen et al. 1992	Glenn 1998[1]

[1]Koi carp.

A Research Approach to Gamete Storage

Fish Maintenance and Gamete Collection

For these experiments, mature male and female koi and goldfish were maintained at the Aquaculture Research Station of the Louisiana Agricultural Experiment Station, Baton Rouge. Only broodstock exhibiting spawning characteristics were selected. Spawning condition was indicated by free-flowing sperm in males, and swelling of the urogenital region in females. Female goldfish were selected for injection based on the presence of rounded bellies, swollen vents and spawning behavior in the presence of males. Female koi were catheterized to assess condition of oocytes. If nuclei were located near the periphery in a majority of the eggs (observed by naked eye), the females were considered to be more likely to respond to hormonal treatment (described below). Fish were not fed for 2 d prior to spawning. For collection of gametes, fish were anesthetized with tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington, USA), and the urogenital papilla was wiped dry. Sperm were hand-stripped and collected into a syringe to avoid contamination with blood and feces.

To stimulate spawning, females received single injections of luteinizing hormone-releasing hormone ethylamide (Peninsula Laboratories, Inc., Belmont, California, USA), at 10 μg/Kg, and of metoclopramide, a dopamine antagonist (Sigma Chemical Corp., St. Louis, Missouri, USA), at 20 mg/Kg (Rothbard 1994). Males did not receive this treatment. Eggs were collected after injection between 9 and 12 hr for koi and 6 to 9 hr for goldfish in a dry bowl, coated with vacuum grease (Dow Corning Corp., Midland, Michigan, USA) to avoid adhesion to the bowl. Eggs were fertilized on separate 100 x 15 mm disposable Petri dishes (Baxter Healthcare Corp., McGaw Park, Illinois, USA) and placed in a recirculating system until percent eyed embryos and hatch were evaluated (koi and goldfish eggs are naturally adhesive when placed into water and activated). Petri dishes were placed in a mesh screen and held vertical to increase oxygen circulation and to avoid sediment accumulation.

Percent Estimation of Sperm Motility

Although subjective, motility is the method most commonly used to evaluate quality of fish sperm, which are typically not motile before dilution in water. Once activated, carp sperm swim rapidly for a short time (usually for 30-40 sec) (Billard et al. 1995). A 2-μL sample of semen from each male was examined using dark-field microscopy at 200-x magnification immediately following activation with 20 μL of deionized water. Percent motility took into account initial movement and duration of the motility within each activated sample. Sperm vibrating in place were not considered to be motile.

Osmolality and Sperm Activation

Osmolality influences the initial activation and duration of sperm motility. By determining the osmolality of body fluids, the environment for non-motile sperm can be better understood. Blood samples from 33 koi (male and female) and 4 goldfish (male) were collected and allowed to clot. Plasma (10 μL) was used to determine osmolality with a vapor-pressure osmometer (model 5500, Wescor Inc., Logan, Utah, USA) to aid in preliminary formulation of extenders. Osmolality was also determined from 10 μL samples of seminal plasma from koi (n = 20) and goldfish (n = 9).

The effect of osmolality on motility was evaluated using dilution of 2-μL sperm samples with 20 μL of graded test solutions (Bates et al. 1996). The solutions were prepared from C-F

HBSS by use of reagent-grade chemicals (Sigma Chemical Corp.): 8.00 g of NaCl, 0.40 g of KCl, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of Na_2HPO_4, 0.06 g of KH_2PO_4, 0.35 g of $NaHCO_3$, 1.00 g of glucose, and sufficient deionized water to yield the desired osmotic pressure. Removal of $CaCl_2 \cdot 2H_2O$ from the standard formulation of HBSS was necessary to avoid a gelatinous condition from forming which suppressed motility as observed in the sperm of razorback sucker *Xyrauchen texanus* (Tiersch et al 1997). Sperm from five koi males were diluted at 1:10 (sperm:C-F HBSS) (305 mOsmol/kg). Test solutions ranged from 50-391 mOsmol/Kg in increments of ~25 mOsmol/kg. Sperm from five goldfish were diluted at 1:7 (sperm:C-F HBSS) (310 mOsmol/kg) prior to the experiment. Test solutions ranged from 18-416 mOsmol/kg in increments of ~20 mOsmol/kg. The osmolality of the sperm activation medium was determined by a 10-μL sample taken immediately after motility estimation from the microscope slide and analyzed by osmometer. Percent motility was used to determine the osmolality at the threshold activation point (defined as 10% motile sperm) and the complete activation point (highest osmotic pressure yielding the highest percent of motile sperm).

Extender Osmolality and Refrigerated Storage

Maintenance of sperm in a non-motile state (allowing activation when diluted in water) for long periods is affected by the osmotic pressure of the surrounding environment. Sperm samples (0.2 mL) from six koi males were aliquotted into 50-mL disposable plastic beakers (B2722-50A, American Scientific Products, Illinois, USA). The extender (C-F HBSS) was prepared at six osmolalities: 209, 245, 270, 308, 357, or 397 mOsmol/Kg and added to the beakers with sperm at a final dilution of 1:7 at a final volume of 1.6 mL. An undiluted control (0.2 mL) was also included for each male. All beakers (42 total) were stored at 4 ^{o}C. Motility was assessed daily until sperm could no longer be activated.

Sperm Dilution and Refrigerated Storage

Sperm dilution with an extender can prolong fertilizing ability through time. Based on the osmolality storage experiment, C-F HBSS with an osmolality of 305 mOsmol/Kg was chosen for this study. Six dilution ratios (1:0, 1:1, 1:3, 1:7, 1:15 and 1:20) were tested. Sperm samples (1.2 mL) from five koi males were collected. A sperm sample (0.2-mL) from each male was added to 4 mL of C-F HBSS (yielding a dilution ratio of 1:20) and was aliquotted into 50-mL disposable plastic beakers. An additional 0.5-mL sample from each male was placed into beakers to serve as undiluted controls. The remaining 0.5 mL of undiluted sperm from each male was used for serial dilutions with C-F HBSS. All beakers were stored at 4 ^{o}C and contained 0.5 mL of sperm-extender medium. Motility was assessed daily until sperm could no longer be activated.

Based on the goldfish activation studies, solutions of C-F HBSS, NaCl, and $NaHCO_3$ were prepared at 310 mOsmol/Kg for use as extenders. The NaCl solution contained 1g of NaCl in 100 mL of deionized H_2O (18 MΩ/ cm; Barnstead Nanopure D4741 ion-exchange system, Dubuque, Iowa). The $NaHCO_3$solution contained 1.5 g of $NaHCO_3$ in 100 mL of deionized H_2O. Sufficient deionized water was added to obtain an osmolality of 310 mOsmol/kg. Sperm (0.1 mL) was aliquotted into 15-mL centrifuge tubes (Baxter Scientific Products, McGaw Park, Illinois, USA; C3920-15). Extender (0.7 mL) was added to centrifuge tubes containing sperm from each of five males. Undiluted sperm (0.1 mL) from each male was used as a control. The first day of storage was designated as “0 d”. The samples were held in a refrigerator at 4 °C, and were mixed by manual inversion at 24-hr intervals. Motility was assessed daily.

Fertilization Capacity with Stored Sperm

Eggs from female koi (n = 2) were hand-stripped into dry, greased bowls and dispensed (0.25 mL) into 100 x 15 mm disposable Petri dishes. One day prior to stripping of eggs, fresh sperm from 6 males was collected and maintained diluted (1:7) with C-F HBSS (306 mOsmol/Kg). On the day of egg collection, fresh sperm was collected undiluted. Sperm-extender media was added undiluted to eggs (0.20 mL) or diluted (0.20 mL) and activated immediately following stripping of eggs. Water (10 mL) at 23 °C was added to initiate motility and activate eggs. Eggs were incubated in a recirculating system at 23 °C. The concentration of spermatozoa of four males was estimated by hemacytometer to be 1.3×10^9 sperm per mL.

Eggs from goldfish (n = 5) were hand stripped immediately following dilution of sperm with extenders (C-F HBSS, NaCl, and $NaHCO_3$), and were placed in 100 x 15 mm disposable Petri dishes. Sperm-extender media (0.02 mL) or undiluted sperm (0.003 mL) was added to 0.1 mL of eggs. Water (10 mL) at 29 °C was added to initiate motility and activate eggs. Sperm-extender media was prepared 30 min prior to stripping of eggs, and was added and activated immediately following collection of eggs. Inseminated eggs were incubated in a recirculating system at 29 °C. The percentage of eyed embryos at 24 h was calculated by dividing the number of eyed eggs (recognizable by black pigmentation) by the total number of eggs in the Petri dish.

Recommended Statistical Analysis

All percent motility values were arcsine-square-root transformed before statistical analysis. The osmotic pressures of blood plasma and seminal fluid were compared using a Student's t-test assuming equal variances (Microsoft Excel 5.0, Microsoft Corp.). In sperm activation with different osmolalities, the threshold activation point was compared to the complete activation point using a paired Student's t-test (Excel 5.0). In the koi osmolality and dilution studies, a repeated measures analysis of variance (SAS 6.10, SAS Institute Inc., Cary, North Carolina, USA) was used to test the effects of osmolality (209, 245, 270, 308, 357, 397 mOsmol/Kg, or undiluted) and dilution (1:0, 1:1, 1:3, 1:7, 1:15 or 1:20) on motility over time. In the goldfish storage study, a repeated measures analysis of variance was used to test the effect of extender (C-F HBSS, NaCl, and $NaHCO_3$) and time on sperm motility. In the goldfish fertilization study, a two-way analysis of variance was used to test the effect of extender (C-F HBSS, NaCl, and $NaHCO_3$) and individual female (n = 5) on percent eyed embryos. For all analyses, Duncan's multiple range test was used to determine if significant differences ($P < 0.05$) existed among treatment means.

Results for Gamete Storage

Osmolality and Sperm Activation

Koi sperm motility decreased as the osmolality of C-F HBSS increased (Figure 1). The threshold activation point (10% motile sperm) occurred at 252 mOsmol/Kg, and the complete activation point (highest observed motility) occurred at 162 mOsmol/kg. Osmolality at complete activation was significantly higher ($\underline{P} < 0.0001$) than at threshold activation. The osmolality that prevented activation was higher than the osmolality of seminal fluid (mean ± SD: 266 ± 19 mOsmol/Kg) but lower than the osmolality of blood plasma (286 ± 7 mOsmol/Kg). The osmotic pressures of these fluids were significantly different ($P < 0.0001$). In the zone of incomplete activation, a reduction of 10 mOsmol/Kg increased motility by ~10%.

Goldfish sperm motility also decreased as osmotic pressure increased (Figure 1). threshold activation occurred at 253 mOsmol/kg, and complete activation occurred at 179 mOsmol/Kg ($P \leq 0.0001$). The osmolality of seminal fluid (253 ± 23 mOsmol/Kg) was lower than the osmolality of the blood plasma (274 ± 5 mOsmol/Kg).

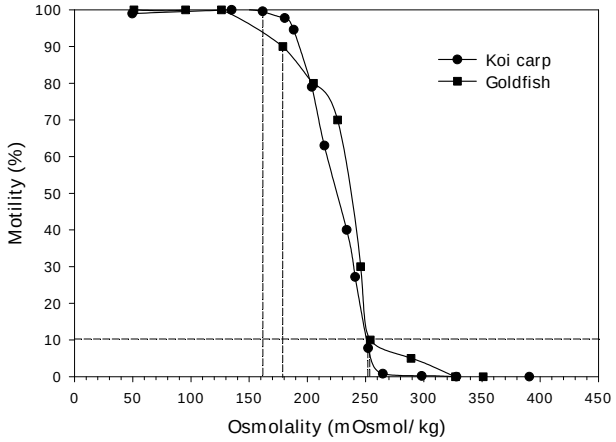

Figure 1. Percent sperm motility of koi (circles) and goldfish (squares) across a range of C-F HBSS osmolalities. Threshold activation is indicated by dashed horizontal lines; complete activation is indicated by dashed vertical lines. Each point represents the mean value for five fish.

Extender Osmolality and Refrigerated Storage

Motility was assessed for 26 d (Figure 2) and the osmolality of C-F HBSS influenced storage time of koi sperm at refrigerated temperatures. At 5 d, the motility of sperm stored at 270, 245, and 308 mOsmol/Kg was significantly higher ($P = 0.0001$) than that of sperm stored undiluted or at 209, 357, or 397 mOsmol/Kg. Samples at 270 mOsmol/Kg maintained motility (>1%) for 19 d. Undiluted sperm lost motility within 3 d.

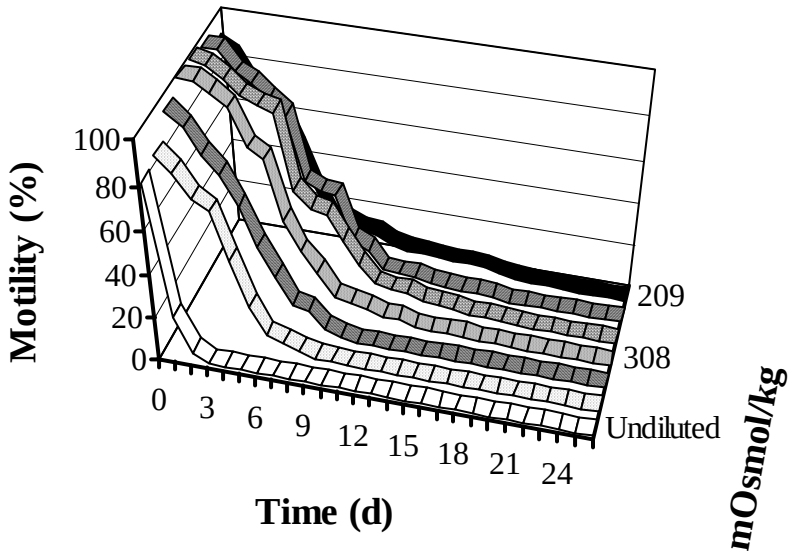

Figure 2. Percent motility of sperm from koi (n = 6) was monitored daily for 26 d. Sperm were stored undiluted or diluted 1:7 in C-F HBSS at six osmolalities. At 5 d, sperm stored at 270, 245, and 308 mOsmol/Kg had significantly higher ($P = 0.0001$) motility.

Sperm Dilution and Refrigerated Storage

Dilution ratios in C-F HBSS did not affect motility of refrigerated koi sperm. Motility was assessed for 12 d (Figure 3), and no significant differences were found over time (P = 0.7640). The dilution ratios of 1:0 and 1:20, although not significantly different, yielded consistently lower motility.

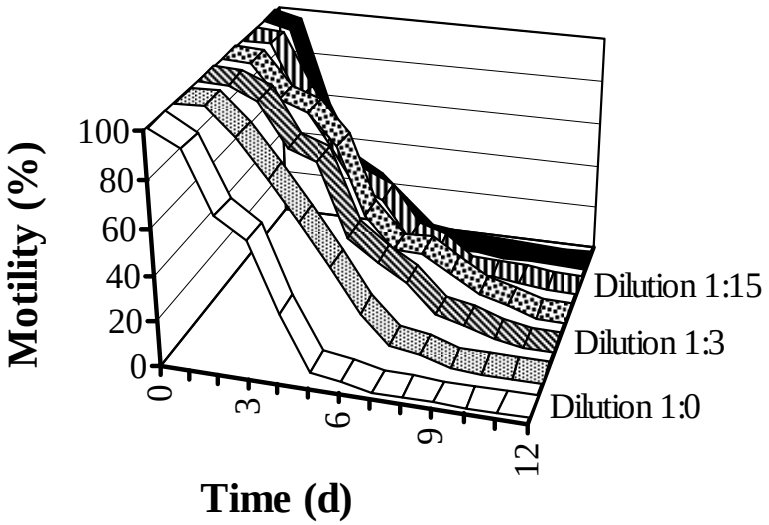

Figure 3. Percent sperm motility of koi (n = 5) was assessed for 12 d after dilution (v:v) in C-F HBSS (305 mOsmol/kg). No significant difference was found among dilutions.

Storage in extenders had a significant effect on goldfish sperm motility (P = 0.0001). Motility of sperm diluted in C-F HBSS at 3 d was 57 ± 52%, although there was much variation at this time: 3 samples had 95% motility and 2 had 0% motility (Figure 4). Motility of sperm diluted in NaCl was 46 ± 32% at 3 d. Motility was not observed in undiluted sperm samples at 4 d. Sperm cells failed to fully suspend in solutions of $NaHCO_3$, and motility ceased within 1 d. No motility occurred in any activated sperm following 6 d.

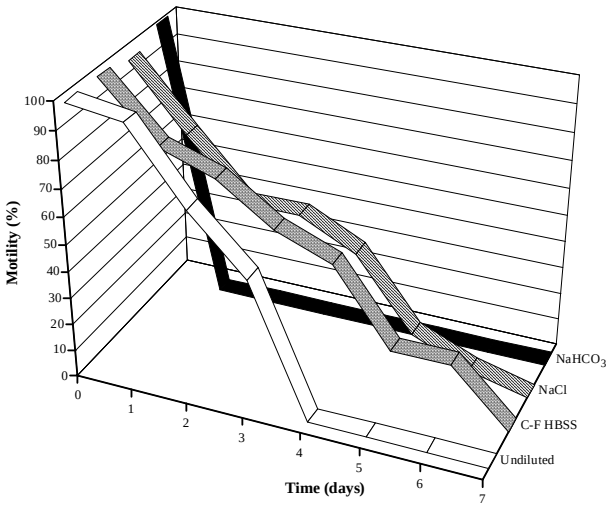

Figure 4. Percent motility for goldfish (n = 5) sperm samples in three extenders (C-F HBSS, NaCl, and $NaHCO_3$) assessed at 24-hr intervals. Samples in C-F HBSS had higher motility after 7 d. Samples in NaCl, $NaHCO_3$, and dry (undiluted) were significantly lower after 7 d.

Fertilization Capacity with Stored Sperm

Fertilization capacity with koi eggs was similar when using undiluted (90 ± 7%) or diluted sperm (92 ± 4%). Fertilization capacity with goldfish eggs was lowest (39 ± 18%) when using sperm diluted with NaCl ($P = 0.0200$). There was no significant difference ($P = 0.1026$) in percent eyed embryos among eggs fertilized with sperm suspended in C-F HBSS (49 ± 21%), $NaHCO_3$ (49 ± 17%), and the undiluted control (48 ± 28%).

Observations for Gamete Storage Procedures

Refrigeration of sperm offers several advantages including use for hybridization and crossbreeding which can be performed in the hatchery. To increase motility and fertilization capacity, an overall evaluation of extenders and their effects on sperm is necessary. As indicated herein, extenders can be evaluated by reviewing the interaction between osmolality and dilution. Blood and seminal plasma can be useful to predict osmolalities at which the extender will maintain non-motile sperm. The osmotic pressure of seminal plasma of koi reported in a previous study was 273 ± 5 mOsmol/Kg (Lubzens et al. 1993). In another study, blood plasma of common carp was reported to be 302 ± 5 mOsmol/Kg and seminal plasma was 302 ± 5 mOsmol/Kg (Morisawa et al. 1983). These values were similar or higher than those from this study, possibly due to different broodstock or environmental influences such as water quality, and indicate that values should be measured for the particular stocks under study.

Sperm activation is an important consideration in the selection and preparation of extenders for sperm storage. Extenders should be prepared at sufficient osmotic pressure to inhibit sperm motility during storage. Sperm of goldfish become motile at spawning because of a reduction of the osmolality (Morisawa et al. 1983). Activation of goldfish sperm should occur at osmotic pressures below that of the seminal plasma (317 mOsmol/Kg). In our study, motility was initiated at osmolalities as high as 271 mOsmol/Kg. The osmolality of extenders prepared for refrigerated storage of goldfish sperm should exceed this value to maintain viability and fertilizing ability over time.

Several studies have reported sperm storage of cyprinids such as the common carp (Sneed and Clemens 1956, Hulata and Rothbard 1979, Saad et al. 1988), silver carp *Hypophthalmichthys molitrix* and bighead carp *Hypophthalmichthys nobilis* (Chen et al. 1992). Various extenders have been used to improve motility and fertilization capacity of refrigerated sperm. Sperm quality is characterized by the ability to fertilize eggs, and subsequent normal development of embryos and fry. Bacterial activity could have reduced sperm motility over time in the present study. Fertilization rates comparable to those of fresh sperm have been maintained in common carp sperm stored at 4 ^{o}C for 16 d with antibiotics and supplemental oxygen (Saad et al. 1988). The present study also show that it is possible to store goldfish sperm in C-F HBSS (310 mOsmol/kg) at 4 °C for as long as 4 d without a significant decrease in motility. Further research is needed to explain why high variation in motility occurred among samples.

Motility of goldfish sperm was retained for 6 d in samples diluted in NaCl, although with a lower rate of fertilization compared to sperm suspended in C-F HBSS, which reduces its value as an extender for extended refrigerated storage. Compensation for low fertilization capacity may be achieved by use of a larger amount of sperm during insemination (Saad et al. 1988). Increased fertilization in common carp was obtained by mixing of sperm and eggs before activation of gametes (Billard et al. 1995), although such methods have not been reported for goldfish. Sperm samples did not remain in suspension in the $NaHCO_3$ solution, making it unsuitable as an

extender. Furthermore, goldfish gamete production occurred in all injected males and 50% of injected females. Intramuscular dosing of metoclopramide and synthetic luteinizing hormone-releasing hormone were used to stimulate gamete production in koi (Rothbard 1994), a method not previously evaluated for goldfish.

Storage of fish sperm in extenders such as C-F HBSS would benefit commercial propagation of koi carp and goldfish. Refrigerated storage is essential for shipping of samples and establishment of cryopreservation programs and germplasm banks for all species. The literature database for this work can be broadly distributed across journals, disciplines and countries. We made our own translations for all of the reviewed studies, but this capability may not be available to all researchers. In addition, a high level of variation in experimental methods and reporting makes direct comparison of results problematic. Thus, decisions on extender choice remain largely empirical for most situations, and currently need to be established on a per-species, per-laboratory basis. This study can provide a general model for short-term gamete storage of freshwater fish species. However, eventual standardization of approach and reporting criteria would greatly facilitate this work and allow cross-laboratory comparisons.

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Appendix. Review of reports addressing refrigerated storage of sperm of common carp *Cyprinus carpio*. Abbreviations: EG, ethylene glycol; DMSO, dimethyl sulfoxide; G, glycerol; PG, propylene glycol.

Extender[1]	Dilution (sperm: extender)	Temperature (^{o}C)	Storage Time (hr)	Comments	Reference
not specified	not specified	0-2, 2-6	200	Motility at 150 h was 100% (0-2 ^{o}C) and 0% (2-6 ^{o}C). Fertilization was >50%. Control had 27% fertilization.	Musselius 1951
FR, PB with 6% glycerin, sodium citrate dihydrate with 6% glycerin	not specified	3-5	720	Stored in 1-mL vials. Decline in motility after 10 d. Phosphate and Na-citrate had motility for >1 wk at 3 ^{o}C. Penicillin (1000 U/mL) interfered with motility.	Sneed and Clemens 1956
egg-yolk-citrate, sodium citrate, PB, Holtfreter's solution plus 1% glycerin, FR plus 1% glycerin	not specified	0-5, 28-33	72	Thawed at room temperature for 5-10 min before estimating motility. Three extenders were unsatisfactory. Sperm in Holtfreter's solution motile for 50 hr at 0 ^{o}C. In water, sperm were motile for 2 min. Sperm in frog Ringer's motile for 72 h at 0-5 ^{o}C. At 28-33 ^{o}C, sperm in frog Ringer's solution were motile for 4.5-6 hr.	Bhowmick and Bagchi 1971
sodium phosphate and yolk	not specified	4	144	Sperm activated with Woynarovich solution. Undiluted sperm gelled after 144 hr. Addition of yolk extended motility (70% at 24 hr). Other combinations of extenders did not aid in sperm storage.	Kossmann 1973
A, mC, C, Ringer's fluid, milk-yolk, ethanol, diluent for freezing bull's semen. 5-20% EG, DMSO, G, or PG was added.	1:1	2, 4	552	Sperm stored in ampoules. Sperm stored in Alsever's solution with EG for 1-19 d at 2 ^{o}C.	Moczarski 1973

[1]See Table 1 for list of chemicals.
[2]Koi carp.

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (^{o}C)	Storage Time (hr)	Comments	Reference
rinsing solution	5:3	0-5	45	Sperm stored in glass tubes. No difference in fertilization or hatch when stored diluted (91%) or undiluted (91%), or with fresh sperm.	Hulata and Rothbard 1979
glucose-yolk diluent	1:0, 1:1, 1:3	2	10-36	Fertilization similar to control. 90% of fry had abnormalities with stored sperm. Stored at 18-25 ^{o}C undiluted for 40 hr maintained motility longer when activated by 5% glucose or sodium diphosphate (pH 8.4) than water (pH 6.8 or 8.4).	Kiselev 1980
not specified	not specified	2-9, ice	3.5	Stored in 1-mL disposable plastic syringes. Motility was 100% at 3.5 hr (2-9 ^{o}C). Motility was 2% at 24 h, For ice, sperm motility was 90% at 2.5 hr.	Withler 1980
not specified	not specified	2-8	216	Motility lasted 180 h. At 168 hr, fertilization (82%) and hatch (75%). Sperm volume:egg number was 200-250,000:1.	Belova 1981
not specified	not specified	2-5	24-48	Stored in beakers and activated by water and Woynarovich solution. No difference in motility or fertilization between cooled and fresh sperm. .	Jahnichen 1981
not specified	not specified	6-8, 2-4	10-12, 120	Significant hydration in semen for prolonged storage. After storage, reduction of fat and protein in the dry matter and change in the ratio of lipid fractions.	Belova 1982

[1]See Table 1 for list of chemicals.
[2]Koi carp

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (°C)	Storage Time (hr)	Comments	Reference
not specified	not specified	4-6	24	During storage, ribonucleic acid content decreased and deoxyribonucleic acid content remained the same.	Nedovesova 1983
saline solution	1:10	4	480	Stored in 5-mL aliquots in 50-mL flasks (0.5 cm thick). Washed or centrifuged before adding extender. Motility without dilution in antibiotics was 0% at 6-8 d. Motility and fertilization with dilution and antibiotics was ~100% at 8 d. Variability was high beyond 8 d. Oxygen improved fertilization for 6 d. Hatch from stored sperm (15 d) not different from fresh sperm. Dilution did not improve motility or fertilization. Extender was used to activate gametes (2.63 g NaCl, 0.37 g KCl, and 2.42 g Tris, per L, pH 8.0). Sperm volume:egg number was 10^{-2} to 10^{-8}:200.	Saad et al. 1988
solution with Na-citrate or NaCl	1:10	0-4	Na-citrate reactivated at 198; NaCl at 15	Higher concentrations of both extenders were used for storage. Na-citrate provided longer duration of motility and storage.	Magyary et al. 1991
solution with KCl and Tris HCl	1:150	2	10	Dealt with potential to move, not motility directly. Seminal fluid was unable to maintain motility. Motility was optimum between 3.73-14.91 g/L KCl. Similar results with NaCl media at 7.31-8.77 g/L.	Redondo-Muller et al. 1991

[1]See Table 1 for list of chemicals.
[2]Koi carp

Appendix. cont.

Extender[1]	Dilution (sperm: extender)	Temperature (°C)	Storage Time (hr)	Comments	Reference
D-15, D-16, D-17, D-19, D-20, or D-21. DMSO was added at 2, 4, 6, 8, 10, 12, or 14%.	1:1, 1:2, 1:4, or 1:8.	2-4	312	Extender, D-19, and 6-8% DMSO gave best results. Optimal dilution was 1:1. Motility at 12 d was 5-15%.	Chen et al. 1992
not specified	not specified	5-9 (~7), 20	5	Sperm stored in 100-mL glass beakers. No difference in fertilization rates between stored and fresh sperm with either egg storage temperature. Volume of sperm to number of eggs was 0.1 mL:200-400.	Rothbard et al. 1996[2]
C-F HBSS	1:0, 1:1, 1:3, 1:7, 1:15, or 1:20	4	288	Sperm stored in 50-mL disposable plastic beakers. No difference in motilities among dilutions was observed. A dilution of 1:7 was easier to evaluate motilities. An activation curve with C-F HBSS showing 10% motility (252 mOsmol/kg) and 100% motility (162 mOsmol/kg). Different concentrations of C-F HBSS (397, 357, 308, 270, 245, or 209 mOsmol/kg) were evaluated to determine optimum osmolality over time. Extender osmolality of 270, 245, and 308 mOsmol/kg had higher motilities at 5 d (total 26 d). Undiluted control lost motility within 3 d.	Glenn 1998[2]

[1]See Table 1 for list of chemicals.
[2]Koi carp

New Chapter

Channel Catfish Pituitary as a Spawning Aid

Christopher C. Green and D. Roger Yant

Introduction

The opportunity exists to better utilize components of the channel catfish *Ictalurus punctatus* carcass to yield a product for artificial propagation of numerous aquaculture species. Channel catfish culture is an economically important industry in the southeastern United States with a total of 230 million Kg processed in 2008 (USDA NASS 2009). Identification of value-added food products from catfish processing has been sought to increase profits and better utilize edible portions of the animal (Min and Green 2008), as total fillet yield for channel catfish is generally reported to range between 34 and 37% and total meat yield between 43 and 45% (Bosworth et al. 2007, Li et al. 2008, Min and Green 2008). Channel catfish pituitary (CP) is a potential product that is currently not collected or utilized widely as a spawning aid. Because channel catfish are regularly processed for food, the ability to incorporate a collection scheme for CP into the process flow adds value to each animal processed.

Catfish production has a significant impact on regional and local levels. On a local level catfish production has been cited as being responsible for nearly 48% of all employment in Chicot County, Arkansas, with even greater indirect effects on the local economy (Engle 2004). In recent times, however, the catfish industry has suffered more than other U.S. livestock industries, a result of the combined effects of catfish import quantities, low value at processing plants, and increases in feed and fuel costs as a percentage of total variable production costs (Hanson 2008). Hybrid catfish production (channel catfish female x blue catfish *Ictalurus furcatus* male) produces an animal that can offset the current negative economic trend which has placed the catfish industry in a vulnerable position. These two species will not hybridize by natural spawning, so commercial production of hybrids requires hormone-induced spawning of females in the hatchery.

Hatcheries rely on the ability to consistently spawn captive broodfish for aquaculture with reproduction serving as the basis of production, utilizing captive stocks under controlled conditions. The first use of freshly extracted pituitaries to induce ovulation in fish was documented 80 yr ago (Houssay 1931, Mathews 1939). As this research developed and assisted hatchery production, the use of fish pituitaries improved and methods were further refined. Due to the wide distribution and introduction of common carp *Cyprinus carpio* this has become the most common pituitary used for fish production in the world (Chaudhuri 1976). A number of exogenous compounds have been used to induce ovulation in channel catfish including: common carp pituitary (CCP)(Sneed and Clemens 1960, Wolters et al. 1981, Busch and Steeby 1990), human chorionic gonadotropin (Sneed and Clemens 1959, Goudie et al. 1992), gonadotropin-releasing hormone analogue (GnRHa) with dopamine antagonists (DA)(Goudie et al. 1992), and luteinizing hormone-releasing hormone analogues (LHRHa)(Busch and Steeby 1990, Lang and Tiersch 2007). Currently, hybrid catfish producers utilize LHRHa and CCP to induce final oocyte maturation and although few publications exist on the use of CP with channel catfish,

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previous research has demonstrated its potential (Sneed and Clemens 1960, Clemens and Sneed 1968).

The production of hybrid fry has dramatically increased from 4 million in 2004 to over 30 million in 2007 (Tucker and Harris 2008). Economic analysis of fry, fingerling, and foodfish production found that while hybrid egg production is more costly than traditional channel catfish operations, the superior qualities of the hybrid demands 1.6 times the capital while resulting in net returns as high as 2.6 times that of channel catfish (Ligeon et al. 2004a,b). As hybrid production increases, the demand for hormones used in the hatchery will also increase. The substantial benefits previously described are responsible for the increased production of the hybrid, as a result, there is an increased demand for carp pituitary and other approved or low regulatory priority hormones used for hybrid hatchery production. Catfish pituitary could be expected to make up for this demand and provide a local product with far greater consistency when compared to CCP, which is purchased from northern areas of the U.S.

Pituitary Collection

As part of our research on this topic, pituitaries were harvested from three catfish processing plants (Heartland Catfish, Greenwood, MS; Consolidated Catfish, Isola, MS; Simmons Catfish, Yazoo City, MS) throughout the winter and spring of 2009. Heads from channel catfish were collected from a conveyor belt within 10 min of death and selected for widths of 9 cm or greater. Head width was measured as the total width of the head across the eyes with subsamples of heads (n = 15) measured for every 150 pituitaries collected to document size selection. A transverse cut through the head was made above the eyes using an electric-powered handsaw to expose the brain (Figure 1). Pituitaries were placed in 75% ethanol in an ice bath immediately after removal from the cranium. Collected pituitaries were decanted after collection and soaked in increasing concentrations of ethanol (75, 95, and 100%) at 12-hr intervals at room temperature by decanting the solvent between each rinse. A final rinse and decanting of 100% acetone was followed by final drying in a glass dessicator, grinding, and packaging in an airtight container.

Figure 1. Selection and removal of pituitary gland from channel catfish.

Collection Characteristics

Head width was monitored closely to report potential variation across season with specific batches. All fish collected were channel catfish of mixed sex with the exception of male broodfish collected on February 27, 2009. Width selection was relatively consistent, with

operators visually selecting heads with an average width of 9 to 10 cm. With the exception of the broodfish collection, head width of heads collected in December, February, and April were not significantly different (Table 2). Post-hoc analysis was performed using the Ryan-Einot-Gabriel-Welsch multiple-range test (REGWQ). An average head width of 9.5 cm resulted in harvesting of 325-350 heads per g of dried pituitary.

Table 2. Channel catfish head width for batches collected in December, February, and April. Different letters denote significant differences within a column ($P \leq 0.05$).

Collection date	Sexes	Average head width (± SD)	Number of Pituitaries
17-December-08	Mixed	9.5 ± 0.8^{a}	2005
27-February-09	Broodstock males	13 ± 1.2^{b}	381
27- February -09	Mixed	8.9 ± 1.0^{a}	771
6-April-09	Mixed	8.9 ± 0.8^{a}	1300

Collection Efficiency

Pituitary collections using a trained crew of individuals harvested an average of 2,468 ± 415 (x ± SD) pituitaries per work day. The average number of heads processed by individual crew members did not vary among crews of 4, 5 or 8 individuals. No significant difference was found between crew size and the average number of heads processed per crew member (P = 0.91). Because of this consistency it appears that one could project the number of pituitaries collected based on crew sizes between 4 and 8 individuals by estimating approximately 450 pituitaries per worker.

On-farm Use of CP

Investigations were conducted at three hybrid catfish hatcheries to compare performance of CP, CCP and LHRHa to produce final oocyte maturation in female channel catfish. All farms utilized a fixed total dose of 10 mg/kg for CP and CCP treatments. Trials were conducted at farms with established hatchery protocols and practices that might have differed slightly, however, data were collected in a consistent manner at all locations under the supervision of the authors. Participating farms will be identified only as Farm A, B, and C for proprietary reasons.

Ovulation was determined as the proportion of females that released eggs when light pressure was applied to the abdomen. Viable embryos were expressed as a proportion of the total number of live embryos to the total number of eggs in three 4-mL samples as determined by counting fertilized and unfertilized eggs with a stereomicroscope at 36 hr-after fertilization. Data recorded on ovulation and fry production were arcsin square-root transformed and assessed using a general linear model ANOVA. Viable embryos at 36 hr were analyzed across treatments using logistic regression with significant differences assessed using ANOVA of arcsine transformed proportions. Post-hoc analysis was performed using REGWQ.

Farm A

Evaluation of CP and CCP were performed on Farm A within three replicate trials consisting of ~ 20 individuals per trial. Due to low numbers of available prepared pituitaries,

different lot numbers of CP and CCP were used among trials. Ovulation and the number of viable embryos were not significantly different for differing pituitary treatments (Table 3).

Table 3. Mean ovulation and embryo viability for catfish pituitary (CP), and carp pituitary (CCP) treatments at Farm A. Different letters denote significant differences among columns.

Treatment	*n*	Ovulation	Viable embryos at 36 hr
CP	62	77%[a]	45%[a]
CCP	64	86%[a]	46%[a]

Farm B

Examinations conducted at Farm B consisted of three replicate trials each containing ~ 30 individuals with consistent lot numbers across treatments. Ovulation was significantly lower in CP treatments when compared to CCP (Table 4). The proportions of viable embryos at 36 hr were not significantly different between treatments of CP and CCP. Although no significant difference in the proportion of embryos hatched was determined, the overall number of fry produced from CCP treatments was higher due to the significantly greater number of females ovulated in comparison to CP treatments.

Table 4. Mean ovulation, embryo viability, and embryos hatched for catfish pituitary (CP), and carp pituitary (CCP) treatments at Farm B. Different letters denote significant differences among columns.

Treatment	*n*	Ovulation	Viable embryos at 36 hr	Embryos hatched
CP	100	56%[a]	72%[a]	54%[a]
CCP	104	83%[b]	73%[a]	51%[a]

Farm C

Comparative examinations of CP, CCP, and LHRHa were completed at Farm C with four replicate trials each consisting of ~ 45 individuals. No significant differences were observed for ovulation from the three treatments (Table 5). The average number of embryos hatched was significantly greater for catfish pituitary treatments when compared to CCP and LHRHa treatments.

Table 5. Mean ovulation and embryos hatched for catfish pituitary (CP), carp pituitary (CCP), and LHRHa treatments at Farm C. Different letters denote significant differences among columns.

Treatment	*n*	Ovulation	Embryos hatched
CP	141	54%[a]	43%[a]
CCP	208	55%[a]	24%[b]
LHRHa	192	52%[a]	19%[b]

Discussion

All pituitaries collected throughout the current study were from channel catfish of mixed sex with the exception of one collection of male channel catfish broodfish. Collections of channel catfish pituitaries with an average head width of 9.5 cm resulted in 325 - 350 heads per g of dried pituitary. It was impossible to verify the sex for individual heads at the time of collection and segregation of sexes could alter the potency of CP depending upon the level of gonad maturation in males or females. Previous findings have indicated significant differences between males and females in the rate of increase in follicle stimulating hormone (FSH) and luteinizing hormone (LH) gene expression throughout gonad maturation in three-spined stickleback *Gasterosteus aculeatus* (Hellqvist et al. 2006).

In general, inedible raw materials from processing plants are perishable and require further processing such as rendering and refrigeration, with rendering usually performed at other processing plants and demanding transportation (Goldstrand 1992). In the current study catfish pituitaries were collected immediately (≤ 10 min) after death. Preliminary work indicates that carp heads stored at 4 °C for 24 hr did not perform differently as a spawning aid in comparison to pituitaries collected immediately after death (Yaron et al. 2009). When collections were 'scaled-up' several thousand heads (2,500 - 4,500) were processed per d. Collection of pituitaries from refrigerated heads could allow for greater efficiency if potency is similar.

Seasonal Effects

A significant drawback in the use of CP and CCP is the potential for LH content to fluctuate from seasonal collections. The influence of season on LH content is of particular concern due to its involvement in final maturation (Slater et al. 1994, Prat et al. 1996, Yaron et al 2009). Examinations on Farm B demonstrated CP collected in early March were not effective in comparison to CCP, resulting in significantly reduced ovulation. Previous examinations of seasonal fluctuations in carp LH content of pituitaries have resulted in recommendations to harvest pituitaries from carp greater than 1 kg within the months of April to May and September to October within Israeli aquaculture operations (Yaron et al. 2009). In female rainbow trout *Oncorhynchus mykiss* the highest proportions of LH within pituitaries was associated with final oocyte growth and maturation and postovulated females (Gomez et al. 1999). The pituitary LH content of female rainbow trout nearly doubled between final oocyte maturation and after ovulation, indicating that pituitaries collected within or after a spawning period could also perform better than ones collected from individuals undergoing exogeneous vitellogenesis. Expression of LH in female channel catfish pituitaries remained low throughout vitellogenesis and increased rapidly prior to ovulation (Kumar and Trant 2004). In the current study LH content was not determined for CP treatments, however, the authors would expect that LH content would be highest in pituitaries collected in April to June, the closest collection to the natural spawning period for this species.

Future Research Studies

Purification

Currently CP and CCP are reconstituted as desiccated and pulverized pituitaries in saline solutions. Isolation of LH within these crude preparations could allow for a more homogeneous product with increased potency. Purification of LH was first documented within rat pituitary

extracts by fractionation with ammonium sulfate, metaphosphoric acid, ion exchange chromatography and gel filtration through sephadex G-100 (Reichert et al. 1969). By similar procedures highly purified gonadotropic hormones from chum salmon *Oncorhynchus keta* were isolated using sephadex G-75 (Idler et al. 1975). The ability to specifically isolate LH from crude pituitary preparations has been documented for several fish species through the use of gel filtration (Ward et al. 1971, Bedi et al. 1982).

Assay Development

In an effort to increase the consistency of CCP applied as a spawning aid, procedures have been developed to process and validate the concentration of LH within pituitary batches (Yaron et al. 2009). Pituitaries are collected and placed in absolute ethanol and stored at -20°C prior to processing. The pituitary is homogenized with potassium phosphate buffers, centrifuged, and the supernatant is lyophilized and stored at 4°C. The resulting extract is highly stable and can be reconstituted and tested to determine the relative concentration carp LH using radioimmunoassays developed from carp LH antibodies (Levavi-Zermonsky and Yaron 1986). The concentration of carp LH in each batch is adjusted for the administration of a standard priming and resolving dose based on previous validation concentrations that produced ovulation and fertilization. Protocols verifying LH activity in CCP have resulted in a more consistent and stable product (Yaron et al. 2009).

Molecular Approaches

Recent research within the field of hormone application and development has focused on the use of recombinant molecular approaches with specific hormone sequences. Numerous advances in understanding of gonadotropins and their receptors have been made within the past 10 yr. Molecular tools have allowed investigators to characterize, quantify and localize FSH and LH within fish species. Channel catfish FSH and LH have been cloned and sequenced (Lui et al. 2001). Receptors for FSH and LH have been isolated and cloned for channel catfish providing the ability to examine actions of the ligands and receptors partially responsible for oocyte maturation (Kumar et al. 2000, Kumar et al. 2001). Although this work is in its early stages, the ability to insert cloned sequences in expression vectors has been demonstrated (Zmora et al. 2007) or is anticipated (Yaron et al. 2009) within research applications.

Conclusions

The results of these on-farm investigations demonstrate that catfish pituitary can be applied as a spawning aid. Seasonal effects on gonad maturation suggest that collection of CP should be made during the months closest to spawning (April - June in Southeastern USA). Pituitaries collected from processing plants in April resulted in the highest percent of ovulating females. Research on future collections of CP from processing plants should include months that directly overlap and precede the spawning season for channel catfish. The current on farm studies utilized a fixed total dose of 10 mg/Kg across all treatments, although the Investigational New Animal Drug (INAD) exemption for CP stipulates that as much as 25 mg/Kg can be applied. In the absence of a simple assay to determine concentrations of active gonadotropins within CP it is conceivable that the dose could be modified within the parameters of the current INAD based on the prior performance of a specific CP batch. Procedures have been developed to validate the content of LH within pituitary batches in an effort to increase the consistency of

CCP applied as a spawning aid (Yaron and Levavi-Zermonsky 1986, Yaron et al. 2009). The use of CP as a spawning aid increases the options available to hatchery producers based on the continued maturation of broodfish within the spawning season, and for hybrid catfish production offers efficiency advantages that can be linked with cryopreserved sperm of blue catfish to yield a strong production platform (Lang et al. 2003).

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New Chapter

Ultrasonographic Monitoring of Channel Catfish Ovarian Development

Noel Novelo, Douglas Kuenz, Christopher C. Green and Terrence R. Tiersch

Introduction

Gonadal development throughout the life cycle of channel catfish *Ictalurus punctatus* from karyogamy during fertilization, through gonadal differentiation, juvenility, and reproductive activity in adults is controlled by genetic factors (Tiersch et al. 1992, Wolters and Tiersch 2004), and the endocrine system in concert with the environment (Silverstein and Small 2004). Ovarian development in mature channel catfish and the physiological processes directing it are directly affected by oocyte development, starting from recruitment of oogonia (12–15 μm in diameter), transitioning to pre-vitellogenic (15–240 μm), and vacuolated (240–650 μm) and vitellogenic (650–3,000 μm) oocytes. These become secondary oocytes, complete the first meiotic division, undergo meiotic arrest at metaphase of the second meiotic division, and are ready to be ovulated and fertilized (Grizzle 1985). Oocyte development and maturation for spawning in channel catfish begins at 2 to 3 yr of age, although most producers use 3-yr-old catfish for induced spawning (Barrero et al. 2007, 2008). Overall, ovarian and oocyte development is complex, involving environmental, hormonal, cellular, and molecular processes leading to ovulation. Various reproductive indices exist for interpreting these interdependent processes, and for assessing the state of ovarian maturity to select channel catfish for induced spawning. These reproductive indices are obtained using invasive and non-invasive methods.

Invasive methods for evaluating ovarian and oocyte development in channel catfish populations have included ovarian catheterization (Markmann and Doroshov 1983), germinal vesicle visualization (Stoeckel 2000), monitoring of serum hormonal profiles, gross examination of the ovary and oocytes, measurement of gonadosomatic index (gonad weight/body weight x 100) (Brauhn and McCraren 1975), and preservation of ovaries for histological analysis (MacKenzie et al. 1989). The most commonly used non-invasive method for assessing channel catfish ovarian development is visual examination of external morphology, which includes monitoring for a soft, rounded, distended abdomen extending past the pelvic fin and a swollen, reddish urogenital orifice (Clemens and Sneed 1957). Other non-invasive methods for identifying reproductive females are direct observation of active spawning behavior (Bates and Tiersch 1998, Phelps et al. 2007, Lang and Tiersch 2007), and measurement of thermal exposure (degree-days) for prediction of spawning in ponds (Pawiroredjo et al. 2008).

Ultrasonography, a non-invasive technology, has been used in as many as 19 fish species for sex identification (Matsubara et al. 1999, Columbo et al. 2004, Wildhaber et al. 2005), and for development of reproductive indices such as cross-sectional ovarian and testes diameter, gonad volume, and egg diameter (Bryan et al. 2007, Wildhaber et al. 2007, Newman et al. 2008) (Table 1, next page). Two catfishes, the Neosho madtom *Noturus placidus* and the African catfish *Clarias gariepinus,* were studied for monitoring the ovarian reproductive condition before and during the natural spawning season (Bryan et al. 2005, Lazlo et al. 2008).

Although ultrasonography has been used to estimate fillet yield in channel catfish (Bosworth et al. 2001), no ultrasound imaging procedures exist for viewing the ovary of channel catfish. This chapter describes ultrasound imaging of ovaries of channel catfish at different stages of gonadal development, and corresponding histological profiles.

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Table 1. The species (n = 19) and references (n = 23) on use of ultrasonography in fish reproduction were listed. These fishes were grouped below into two main categories (i) freshwater, and (ii) marine and anadromous, according to family, genus and species, with corresponding citations. It was possible to view ovaries and testes in 85% of the species listed.

Common name*	Scientific name*	Citation
Freshwater		
Stellate sturgeon	*Acipenser stellatus*	Moghim et al. 2002
Shovelnose sturgeon	*Scaphirhynchus platorynchus*	Colombo et al. 2004, Wildhaber et al. 2005, 2007, Bryan et al. 2007
Pallid sturgeon	*Scaphirhynchus albus*	Wildhaber et al. 2005, Bryan et al. 2007
Neosho madtom	*Noturus placidus*	Bryan et al. 2005
African catfish	*Clarias gariepinus*	Lazlo et al. 2008
Murray cod	*Maccullochella peelii*	Newman et al. 2008
Marine/Anadromous		
Pacific herring	*Clupea pallasi*	Bonar et al. 1989
Atlantic cod	*Gadus morhua*	Karlsen and Holm 1994
Barfin flounder	*Verasper moseri*	Matsubara et al. 1999
Atlantic halibut	*Hippoglossus hippoglossus*	Shields et al. 1993, Martin-Robichaud and Rommens 2001
Winter flounder	*Pseudopleuronectes americanus*	Martin-Robichaud and Rommens 2001
Yellowtail flounder	*Limanda ferruginea*	Martin-Robichaud and Rommens 2001
Haddock	*Melanogrammus aeglefinus*	Martin-Robichaud and Rommens 2004
Atlantic salmon	*Salmo salar*	Mattson 1991
Coho salmon	*Oncorhynchus kisutch*	Martin et al. 1983
Rainbow trout	*Oncorhynchus mykiss*	Evans et al. 2004a,b
Striped bass	*Morone saxatilis*	Blythe et al. 1994, Will et al. 2002, Jennings et al. 2005
Red hind	*Epinephelus guttatus*	Whiteman et al. 2005
Broadnose sevengill shark	*Notorynchus cepedianus*	Daly et al. 2007

*According to Nelson et al. 2004.

Ultrasound Procedures for Viewing of Channel Catfish Ovaries

The natural spawning season for channel catfish in Baton Rouge, Louisiana (30º 22' 3.3" N, 91º 10' 54.1" W) typically starts in mid-late April when ambient water temperatures remain within a range (21–30 ^{o}C) conducive to spawning (Lang et al. 2003, Pawiroredjo et al. 2008), and continues through May and sometimes into July (Bates et al. 1996). Adult (3–4 yr old) female channel catfish were held in 0.1-acre ponds with blower-driven aeration, and were sampled during early, middle, and late periods of the natural spawning season in 2008. Fish were captured by seining the ponds, and were held in concrete raceways at a salinity of 5 ppt (solar salt, Cargill Inc. Minneapolis, Minnesota, USA) to reduce osmotic imbalances due to stress for 1-3 d before ultrasound imaging and ovary sample collection for histological processing.

Channel catfish were caught with polyethylene dip nets from the concrete raceways, and placed in a portable, 49-L cooler (Igloo, 52 quart Sportsman™) filled with water. The fish maintained an upright swimming position (ventral recumbency), while the left side was scanned anterior to the base of the pelvic fin, and posterior to the dorsal fin (Figure 1). B-Mode ultrasound images were obtained using a laptop-computer ultrasound unit (TELAVET 1000, Classic Medical, Tequesta, Florida) with a multi-frequency (5–8 MHz) linear probe (model LV7.5/60/96Z) set at 8 MHz. During the entire procedure, the probe and the catfish were completely submersed in water, which provided the sole ultrasound transmission medium. The location of the ovary and the largest cross-section were determined by scanning the left side of the abdomen between the pectoral and dorsal fins with the probe, with the probe tip aligned to the bottom (ventral) side of the fish, and the cable end of the probe aligned to the top (dorsal) aspect of the fish. Ultrasound images were recorded on the hard drive of the laptop computer, with each image labeled with the corresponding identification number (i.e., Floy tag) of the fish.

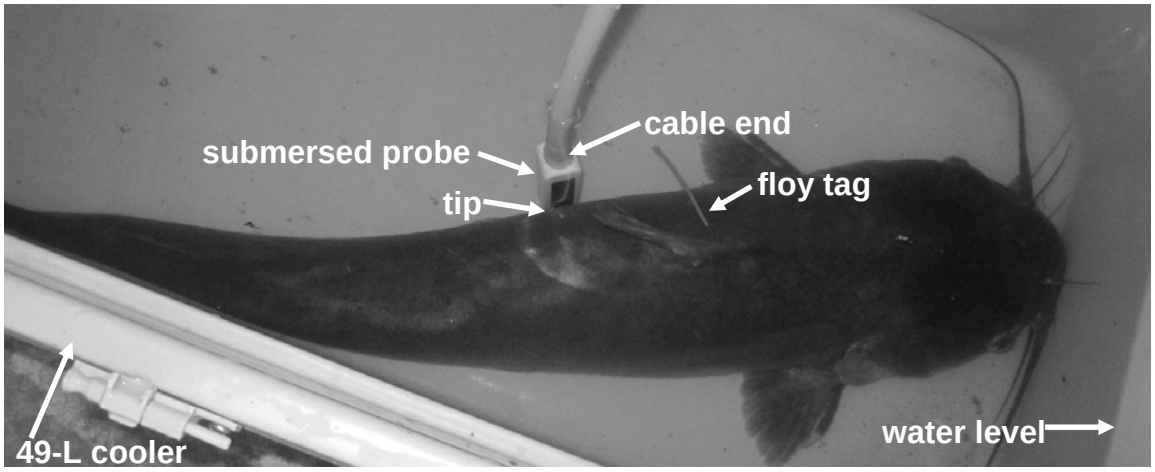

Figure 1. For viewing, the channel catfish were completely submerged in water, with the ultrasound probe placed on the left side, the cable end (connected to the ultrasound unit) located dorsally (adjacent to the spine), and the probe tip located ventrally (adjacent to bottom of the abdomen).

B-Mode ultrasound images of the ovary were created with the emission of ultrasound waves (in this case, 8 million cycles of ultrasound waves per sec) by piezoelectric crystal elements inside the linear array probe. These ultrasound waves were transmitted into the water, which acted as a transmission medium (a similar function is served by application of ultrasound

gel to eliminate the air interface between the probe and the surface of the anatomy being scanned). The emitted acoustic waves made contact first with the skin, and subsequently with muscle and ovarian tissues in the area at which the probe was positioned (Figure 1). The return of these ultrasound waves to the probe was displayed in a rectangular, two dimensional gray-scale image on the laptop monitor. The ultrasound echoes were recorded as dots along a vertical axis, with the dots located in the near view field of the image (top of the display image) creating ultrasound images of anatomical structures (skin and muscle) closest to the probe, and the dots in the far view field of the image (bottom of the display), creating ultrasound images of anatomical structures (ovarian structure) furthest from the probe (Figure 2).

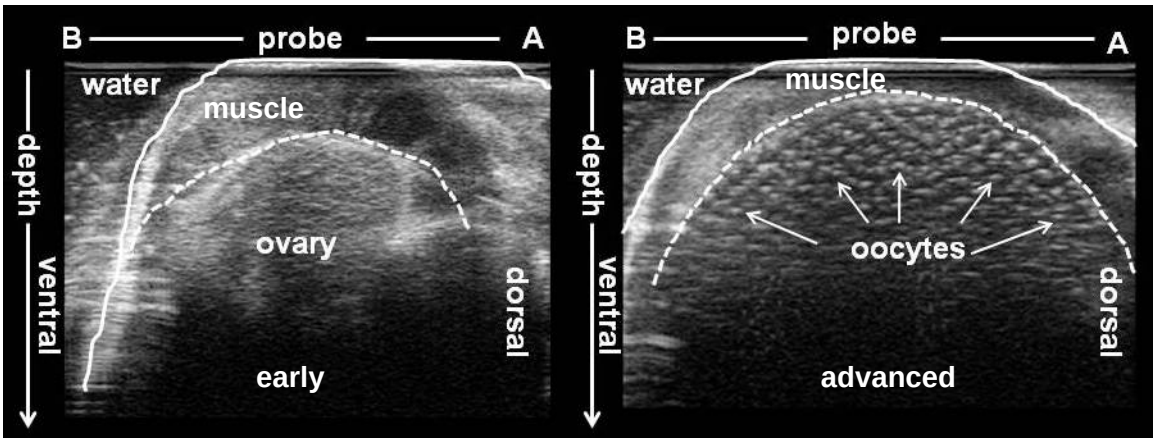

Figure 2. The image produced by a linear array probe is a rectangular, gray-scale ultrasound image, with the top of the image corresponding to the position of the probe. The cable end of the probe (A) corresponds to the top of the fish (right side of the image), and the tip of the probe (B) corresponds to the bottom of the fish (left side of the image). The top of the image (near field) corresponds to anatomical structures closest to the probe, with the skin depicted by the outermost solid line delineating the curving external perimeter of the fish, followed by muscle tissue, the ovary (dashed curved line), and oocytes (indicated by arrows). The image on the left represents early ovarian development in channel catfish, and the image on the right a more advanced stage.

The position of the dots along the vertical axis of the ultrasound image display represents the depth (mm) of the internal anatomical structures from which the echo originated. The brightness of the dot is proportional to the strength of the returning echo, and corresponds to an intensity within a 256 gray-scale range, with the brighter grays representing echoes of greater intensity. These vertical axis lines, when aligned, represent parallel scan lines produced by acoustic pulses and echoes at different points on the linear array of elements, which are rectangular in shape, arranged in a straight line, and produce a cross-sectional gray-scale image of the tranverse scanning plane of the ovary.

Thus, one of the key elements in interpreting the ultrasound image irrespective of gonadal condition of the fish is to understand the relationship of the physical position of the probe on the external anatomy of the channel catfish (Figure 1), and the corresponding probe and anatomical structures in the resulting display image (Figure 2). When the probe was placed on the lateral aspect of the abdomen (Figure 1), the orientation of the ultrasound image in the monitor was displayed with the top of the image (the near field view) representing the nearest distance to the probe, and with the bottom of the image (the far field view) representing the furthest distance from the probe. In these ultrasound images, the cable end corresponded to the right side of the

image (dorsal aspect of the fish), and the tip of the probe corresponded to the left side of the image (ventral aspect of the fish) (Figure 2), but this orientation of the fish anatomy can be switched by using the ultrasound software controls such that the left side of the image corresponds to the dorsal aspect of the fish and the right side of the image corresponds to the ventral side of the fish. The relationship of the orientation of the probe with respect to the external anatomical positioning of the probe and the internal anatomy of the fish should be clearly defined for basic interpretation of ultrasound images (Figure 3).

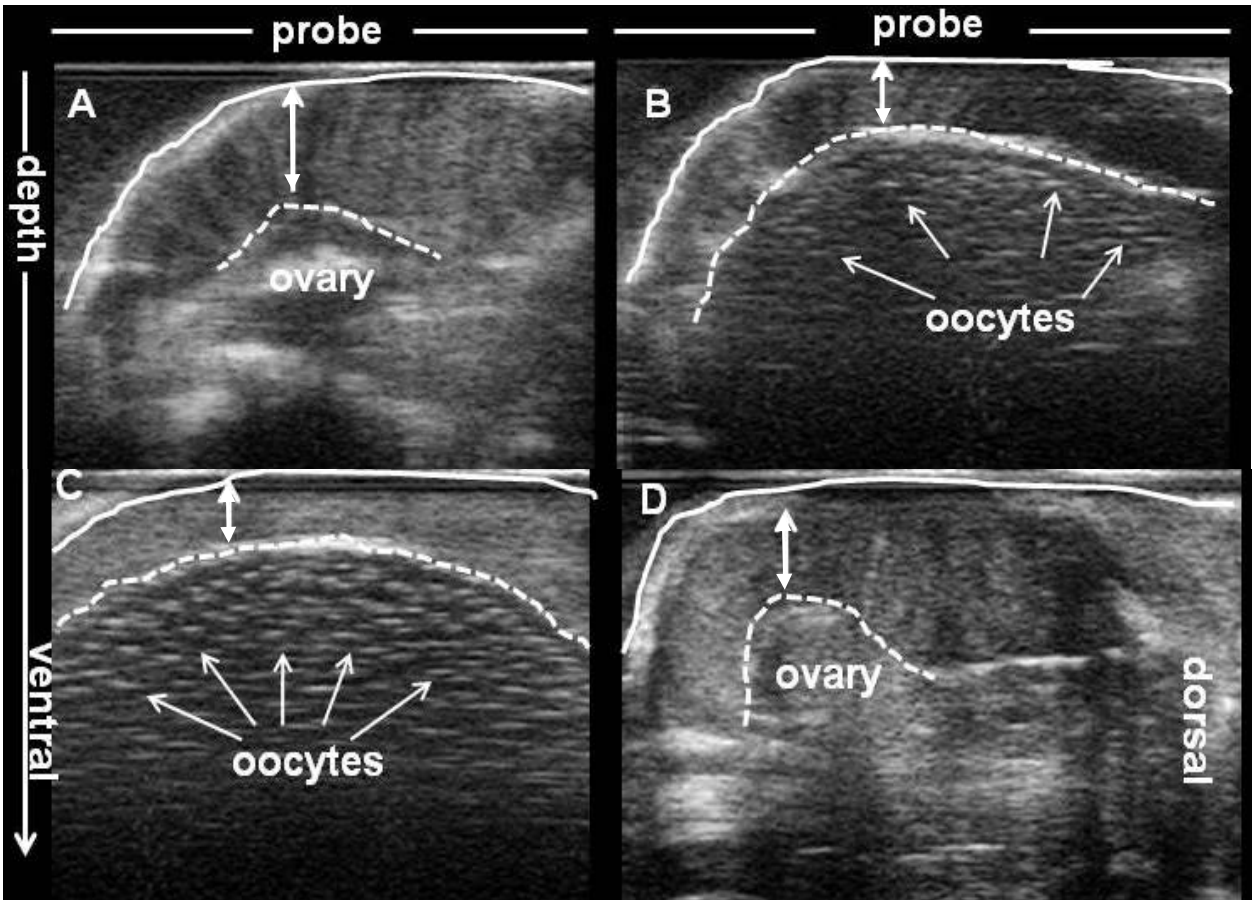

Figure 3. Ultrasonography provided direct images of channel catfish ovaries during the natural spawning season of channel catfish, revealing distinct gonadal appearances for early (A), developing (B, C) and atretic (D) ovaries. The curved solid white line depicts the skin, the anatomical structure closest to the probe. The dashed line depicts the contour of the ovary, with the dorsal and ventral aspect of the fish on the right and left side of each image, and the double-headed arrows between the skin and the ovary showing the changes in thickness of the body wall in each image. Oocytes appear flattened rather than rounded probably due to the polar distributions of aqueous and lipid compartments.

Early gonadal development (Figure 3A) was seen with more frequency early in the natural spawning season (i.e., early April), with ultrasound images displaying a small ovarian size. At this time the shape of the ovary was frequently not clearly defined, or not clearly distinguished from surrounding internal structures, and there were no visible oocytes. The

distance of the abdominal muscle between the periphery of the ovary and the skin (the body wall) was at its widest point.

Ovarian growth was noticeable during the middle and late period of the natural spawning season (i.e., late April to early July), with ultrasound images displaying a progressively enlarging ovarian size (Figure 3B and C). The ovarian structure was immediately visible and did not require multiple abdominal scans to be identified. The shape of the ovary was clearly visible, with the outermost periphery of the ovary curved and clearly defined, similar to the skin margin of the fish which was always visible during ultrasound scans. Oocytes were immediately visible during ultrasound scans. Individual oocytes and a high degree of organization within the ovary were discernible as the spawning season and gonadal growth advanced. The thickness of the body wall between the periphery of the ovary and the skin progressively narrowed with increased ovarian growth.

Towards the end of the spawning season (i.e., July), ultrasound images revealed gonads that were drastically reduced in size, and undergoing atresia (Figure 3D). Atretic ovaries could be identified by images displaying a small, disfigured ovarian wall, and disorganized, disintegrating oocytes that lacked a clear perimeter and regular shape. The body wall thickened with the reduced size of ovaries undergoing atresia.

Histological Profiles of Ultrasound Images

Fish were placed into a lethal dose of MS-222 for removal of ovaries, which were preserved in 10% neutral buffered formalin (NBF). After storage (≥ 1 month), the ovaries were sectioned through the largest cross-section, corresponding to the position of the ultrasound probe, and sent for histological processing to the Histology Laboratory of the Louisiana State University School of Veterinary Medicine, Baton Rouge, Louisiana. The section widths of the ovary samples on the histology slides were 3 – 4 μm, and the chemical stains used were hematoxylin and eosin. Digital images of histology slides were obtained using a stereoscope (Nikon SMZ-U, Tokyo, Japan). A mm-increment ruler was positioned in the upper left corner of each histology slide to provide a standard size reference.

Histology profiles (Figure 4) corresponding to the ultrasound images collected during the spawning season (Figure 3) revealed the microscopic biological progression of ovarian development which was not visible in the ultrasound images. Ultrasound images of early and atretic ovaries (Figure 3A, D) depicted small ovaries with no visible oocytes (Figure 3 A), or small ovaries with disfigured oocytes (Figure 3 D). In contrast, the histology profiles of early and atretic ovaries revealed a large ovarian cross-sectional area, with numerous oocytes (Figure 4A) and distinct primary and atretic oocytes (Figure 4D) enclosed in a thick ovarian wall. The larger ovarian cross-sectional area depicted in the histology profiles was directly related to the sampling of small ovaries, corresponding to early-developing or regressing ovaries (Figure 3 A, D). Ultrasound images of developing ovaries (Figure 3 A, D) depicted a larger cross-sectional area occupied by enlarging ovaries and visible, enlarging oocytes. In contrast, the histology profiles of developing ovaries (Figure 4 B, C), prominently displayed a large oocyte cross-sectional area, rather than a large ovarian cross-sectional area. Consequently, internal oocyte processes such as the formation and coalescence of yolk globules (Figure 4 B, C) were depicted.

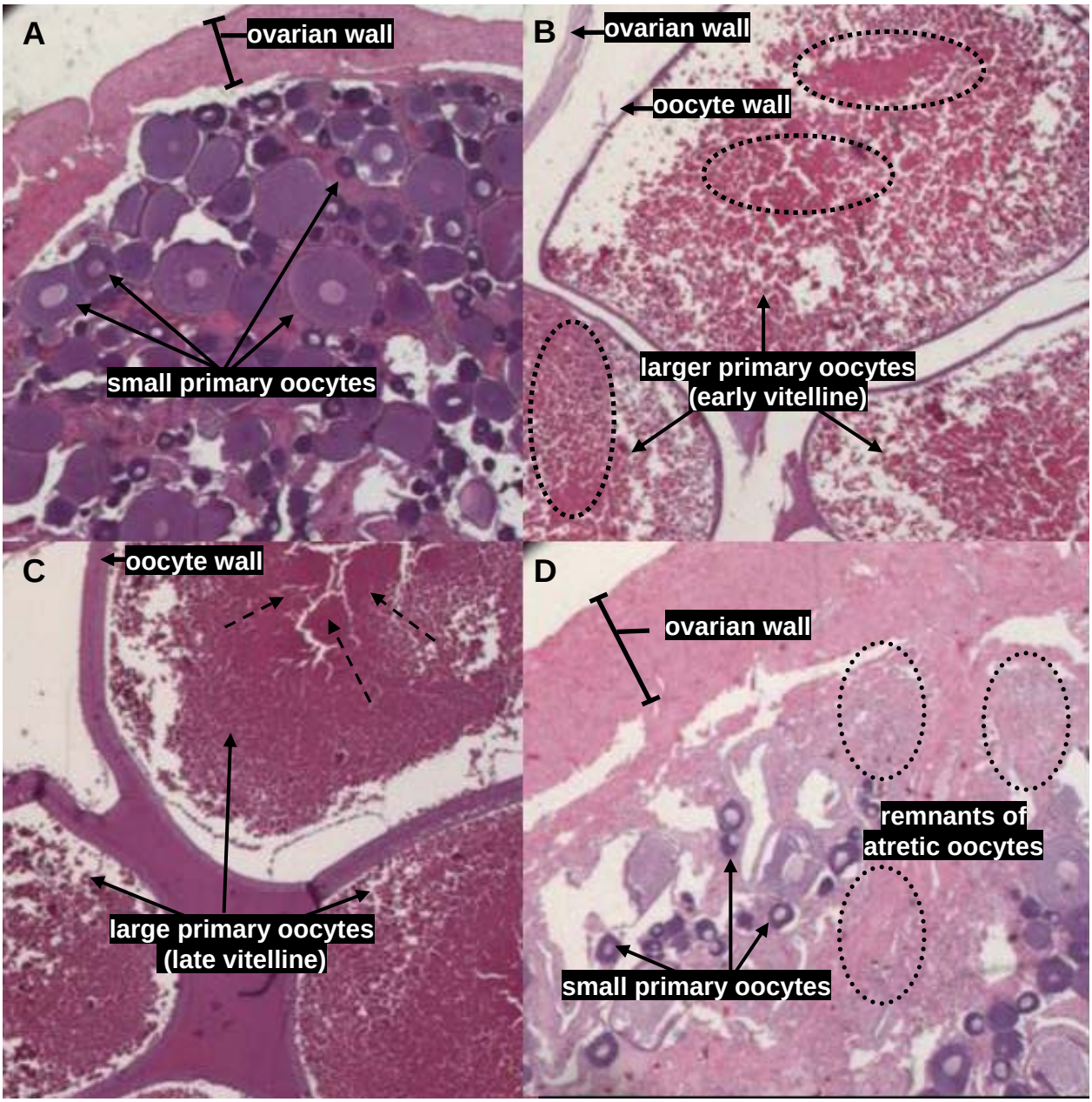

Figure 4. Histology corresponding to ultrasound images of early (A), developing (B, C) and atretic (D) ovaries of channel catfish during the natural spawning season revealed distinct profiles. Histology corresponding to early (A) ovarian development displayed a thick ovarian wall enclosing numerous small primary oocytes with no discernible wall. Histology of developing ovaries (B, C) displayed large vitelline oocytes with a thin ovarian wall, a visible, thin oocyte wall (B), yolk globule formation (B, dotted oval shapes), a thickened oocyte wall (C), and coalescing yolk globules (C, dotted arrows). Histology of atretic (D) ovaries showed remnants of atretic oocytes, and few small primary oocytes.

Conclusions

Application of ultrasound technology provides a direct, non-invasive, visualization method that can be used for evaluation of the reproductive condition of channel catfish females during the spawning season. Histological profiles corresponding to ultrasound images revealed microscopic processes that were not visible with ultrasonography, but which corroborate ultrasound imaging of ovarian development, demonstrating a strong potential utility of ultrasonography in channel catfish reproduction. Linking the ultrasound images with histology of gonadal development provides a comprehensive view of ultrasound images representative of different gonadal stages ranging from developing and developed, to advanced and atretic ovaries.

The ability to use ultrasound technology and corroborate its application with histology and other biometric indices is important in understanding the biological development of the channel catfish ovary. Identification of females in late vitellogenesis is critical for efficient hormonal induction of spawning in the hatchery. This is especially important in the application of cryopreservation to the production of hybrid catfish (channel catfish females x blue catfish *Ictalurus furcatus* males) at a commercial scale. To adequately assess and improve cryopreservation of aquatic species, technologies for understanding gonadal biology need to be incorporated into selection of females with the highest chance of ovulating fertilizable oocytes. Further studies are needed for addressing qualitative and quantitative analysis of fish gonadal development based on ultrasonography and histology to fully explore the potential application of ultrasound as a standard, non-invasive, informative means of commercial-scale assessment of a variety of fish species.

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II. Gamete Quality

New Chapter

Sperm Quality Evaluation for Broodstock Improvement

Elsa Cabrita, Vanesa Robles, Carmen Sarasquete and Paz Herráez

Introduction

Sperm quality has been a focus of research in several fields and can be used as a biomarker for the assay of male status. Until now not much attention has been paid to the relationship between sperm quality and other male characteristics, ignoring the importance of knowing what is "behind spermatozoa". In the present chapter we have tried to summarize the principal methods of fish sperm analysis, to give a survey on the relationship between sperm quality and several biological factors and to suggest how to benefit from that knowledge to improve male broodstocks.

The Importance of Sperm Quality Assessment

Several characteristics have been used to determine sperm quality. Standard analysis may include parameters such as spermatozoa concentration, subjective assessment of motility, sperm volume, seminal plasma osmolarity and pH, which will give a clue to quality in a given species and could be important steps for the first characterization of sperm. However, good characterization requires evaluation of certain aspects of cell function that may be impaired (Table 1, next page). Sperm quality can be assessed by its constituents: seminal plasma and spermatozoa. Basic studies on seminal plasma constituents and its variation such as enzymes (i.e., lytic, oxidative, and metabolic), metabolites, sugars, vitamins, amino acids, lipids, fatty acids, glucose, lactate and other inorganic compounds can provide useful information and have been developed in several species and in several approaches, from cryopreservation to toxicology studies (Kime et al. 2001, Ruranwga et al. 2004). These determinations can detect the lack or loss of a certain compound as well as alterations in cell integrity and metabolism.

Spermatozoa functions have also been the focus of attention as markers for sperm quality. Sperm need to acquire and maintain their motility capacity until they reach the oocyte. Thus, motility and energetic metabolism have been the most studied parameters and several correlations have been achieved with the fertilizing capacity of sperm (e.g., Lahnsteiner and Patzner 1998). Currently, sperm motility can be well characterized in terms of velocity and motility pattern using computerized sperm analysis systems reviewed elsewhere in this volume. But sperm, apart from reaching the egg, need to recognize and fuse with the oocyte, as well as contribute to embryonic development, so other cellular characteristics should also be evaluated to explain milt fertilization ability.

One regularly used parameter is cell viability, which is mainly based on the analysis of cell membrane integrity and functionality. Most assays describing cell viability are performed with the use of permeant or impermant dyes (often fluorescent probes) which are included or excluded from the cytoplasm according to the plasma membrane status. The need to assess the effects of toxicant exposure and cryopreservation procedures has greatly contributed to the

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Table 1. Examples of assays that can be considered for the analysis of sperm quality in aquatic species.

Assay	Structure assayed	Reference
Mitochondria Functionality		
Fluorescent dyes (JC1, Rh123, MTT assay)	Mitochondria	Ogier de Baulny et al. 1997, Hamoutene et al. 2000
Cell Viability		
Fluorescent and non-fluorescent dyes (Hoecht, PI/SYBR-14, acridine orange, DAPI, trypan blue)	Plasma membrane	Cabrita et al. 2008
Lytic enzymes (acid phosphatase, alkaline phosphatase, ß-D-glucuronidase, protease)	Seminal plasma, sperm	Lahnsteiner et al. 1998
Metabolic enzyme (malate dehydrogenase, lactate dehydrogenase, aspartate aminotransferase, adenosine triphosphatase)	Seminal plasma	Lahnsteiner et al. 1998
Oxidative Stress		
Anti-oxidative enzymes (catalase, glutathione peroxidase, superoxide dismutase)	Seminal plasma	Pagl et al. 2006
Anti-oxidant potential (vitamins E, C, albumin, glutathione, taurine)	Seminal plasma	Cohen et al. 2007
Lipid Peroxidation		
Free radicals, TBARS assay, 8-isoprostane level Fluorescent dyes (Bodipy-C11)	Seminal plasma	Khosrowbeygi and Zarghami 2008, Brouwers et al. 2005
DNA Fragmentation		
Comet assay, apoptosis assays, SCSA, OxiDNA assay	Chromatin	Cabrita et al. 2008, Zhang et al. 2008
Spermatozoa Motility		
Computer-assisted sperm analyzer	Sperm	Martinez-Pastor et al. 2008, Cosson 2007
ATP Metabolism		
ATP and ADP levels, creatine phosphate, adenosine triphosphatase, adenylate kinase	Seminal plasma, sperm	Lahnsteiner et al. 1998
Spermatozoa Morphology		
Electron microscopy, ASMA	Sperm	Marco-Jiménez et al. 2006
HOS-Test		
Hyper or hyperosmotic test	Plasma membrane	Cabrita et al. 2008
Fertility assay	Sperm	Cabrita et al. 2008

Abbreviations: JC1:fluorophore 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolylcarbocyanine iodide; Rh123:rhodamine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; C11:Bodipy- fluorescent dye 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; SCSA:sperm chromatin structure assay; OxiDNA:FITC-conjugate that binds to 8-oxoguanine; TBARS: thiobarbituric acid reactive substances; ASMA:Sperm Class Analyzer.

development of more precise cytophysiological analysis. Mitochondrial status can be analyzed using different specific probes, able to discriminate between active and inactive mitochondria. Many efforts have recently been made in study of DNA structure using different approaches such as the comet assay (single-cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase-nick-end-labelling), SCSA (sperm chromatin structure assay) and the analysis of specific DNA sequences using quantitative PCR, because damage to DNA can impair fertility or embryo development (see methods description in Dmitrieva and Burg 2007).

From the study of motility using more or less sophisticated approaches to the analysis of DNA integrity, the choice and combination of parameters depends on the objective of the evaluation, as well as on available equipment and experience. Quality assessment should be relatively simple for routine use in fish farms, but should be more precise for experimental purposes. It is also important to remark that sperm is not a homogeneous mixture of cells and plasma, but a pool of cells originating from different spermatogonia. Each spermatogonium produces spermatozoa with a different haploid genotype, maturation stage and characteristics. This is the reason why new trends in sperm evaluation emphasize the analysis of spermatozoa subpopulations for some determinations, finding concerns for the use of average values to characterize sperm sample (Martinez-Pastor et al. 2008).

The Importance of Knowing Male Background

Sperm Quality and Genetic Characteristics

It has been assumed that "good" males (high quality sperm producers) would also produce high quality offspring in terms of sperm characteristics, because the transmission of nuclear genes that encode for sperm characteristics would be inherited (Evans and Simmons 2008). This assumption, if true, would be useful in the selection of certain lines of breeders that produce high quality sperm. A number of recent studies have questioned this premise and the question whether maternal and cytoplasmic genes may participate in sperm structure and function has arisen. Work on brown trout *Salmo trutta* did not find a correlation between high quality males and "good genes", but the size and age of fish directly influencing the quality of sperm (Jacob et al. 2007). This fact does not diminish the importance of male genetic background in the definition of sperm quality, but from this and other related studies, it seems that other factors may have a key influence. Although studies of this kind are scarce in fish, as far as we know, recent reports on bird and insect sperm demonstrate that the effects on sperm function of cytoplasmic (mitochondrial) genes, which are maternally inherited, may similarly constrain sperm quality (Gemmell et al. 2004, Zeh and Zeh 2005, Evans and Simmons 2008). Mitochondria are known to play a role in regulating sperm motility in birds (Froman and Kirby 2005) and sperm motility and fertility in mammals (Moore and Reijo-Pera 2000, Gallon et al. 2006). Recently, sizeable cytoplasmic mtDNA effects were reported for sperm viability of the seed beetle *Callosobruschus maculates* but no effect of these genes was found for the morphology of the sperm flagella, which was controlled by nuclear genes (Dowling et al. 2006).

Other strong evidence that maternal genes may contribute to the success of fertilization comes from the experiments performed in chicken *Gallus gallus domesticus*. In this species, sperm motility is under the control of autosomal genes and maternal-derived genes (Froman et al. 2002), almost certainly mtDNA. The role of mtDNA in regulating sperm performance in terms of motility was explained by the fact that motility depends on sperm oxygen consumption, which in turn is dependent on mitochondria, and that immotile sperm were associated with

aberrant mitochondria. Knowing this, it has been possible to select lines with low and high motility sperm and check on those populations that mitochondria function was divergent (Froman and Kirby 2005). As stated before, there are no similar reports in fish sperm; however variations in sperm quality could also be attributed to their nuclear and mitochondrial genetic profiles, with not only paternal background being important, but also the maternal contribution.

Reproductive Performance

Sperm quality can be used to assay the reproductive performance of any individual. Depending on the species, social status and fish reproductive behavior could play a crucial role in the determination or selection of "good males". Sperm competition is one of the features that increases variability within males, leading to a differences in the quality of sperm. Sperm features such as density (sperm counts), spermatozoa morphology (e.g., flagella length) sperm velocity, motility, and so-called "longevity" in some reports (viability) are the principal characteristics affected by sperm competition and can be modified according to its degree (Petterson and Warner 1998, Taborsky1998, Locatello et al. 2006, Rudolfsen et al. 2006, Kaspar et al. 2008).

Hence, population structure should not be ignored, but despite the large number of reports analyzing sperm quality in different fish species, reproductive behavior has been systematically neglected in most approaches. This fact has probably contributed to the intraspecific variability observed between males maintained in the same breeding conditions and even between ejaculates from the same male, more than other background factors (e.g., genetics). Sperm competition is part of the reproductive performance of several fishes and could be important to "increase" sperm quality or select the best males. In some cultured and ornamental species (e.g., rainbow trout *Oncorhynchus mykiss*, common carp *Cyprinus carpio* Atlantic salmon *Salmo salar*, and guppy *Poecilia reticulata*) this could be a possible explanation for intra-individual changes in sperm quality and could be used for male improvement by selection of good quality males (associated with high quality sperm) (Gage et al. 2004, Locatello et al. 2006, Fitzpatrick and Liley 2008, Kaspar et al. 2008).

In Atlantic salmon, sperm velocity was correlated with competition and is considered to be a good predictor of sperm capacity for fertilization when equal numbers of spermatozoa per male are used in a pool (Gage et al. 2004, Wedekind et al. 2007). In the guppy, it was found that sperm viability and motility were higher in high quality males (Locatello et al. 2006). However in common carp, although sperm characteristics such as spermatocrit, motility, and velocity could influence sperm fertilization ability, none of them could explain the variability in sperm quality associated with competition among males (Kaspar et al. 2007, 2008). Thus, when analyzing sperm quality in a species all these issues must be taken into account because they may help in the establishment of appropriate breeding conditions in aquacultured species, and their management could improve sperm quality.

Male Provenance

The source or origin of males is one of the determining factors involved in the quality of sperm. In most of the species recently introduced for aquaculture as well as in those species showing reproductive difficulties in captivity, breeders come from the wild and the assessment of sperm quality is sometimes difficult to interpret. Firstly because male life-history is unknown (e.g., age, reproduction physiology, habitats, breeding season, diseases) and secondly, because in some species there is not enough knowledge to be sure that breeders were properly maintained

and that sperm were collected in the right period of the reproductive season. It is well known that keeping fish in stressful conditions can affect sperm quality and thus produce abnormal gametes. For example, several reproductive dysfunctions have been identified in flatfish males, such as yellowtail flounder and turbot (Zohar and Mylonas 2001). Males from broodstocks captured from the wild during the reproductive season can produce milt with non-mobile sperm (Berlinsky et al. 1997) or milt with high viscosity that does not mix readily with water during egg fertilization (Vermeirssen et al. 1998, 2000).

Another problem occurring with wild stocks is that depending on the species and place of capture, there is no information about the previous reproductive performance of those individuals and whether they were exposed to contaminants during their life cycle in the wild. Most contaminants affect reproduction by direct impairment of gonadal development and germ cell differentiation or by affecting sperm characteristics, thus reducing sperm quality. Heavy metals, organochlorides, organophosphates, carbamates and a range of industrial chemicals can act as endocrine disruptors, affecting sperm motility, velocity, viability, and fertility in a wide range of species (Kime 2001). These agents could produce structural damage to the head or flagella, functional impairment of the energy-producing mitochondria, DNA damage, or developmental abnormalities in sperm structure caused either by exposure of developing sperm or by abnormal testicular development resulting from early life or maternal exposure to pollutants or hormones (Kime et al. 2001). Deleterious effects of tributyltin on sperm motility, viability, and metabolic activity were reported in African catfish *Clarias gariepinus* (Kime et al. 1996, Rurangwa et al. 2002), and heavy metals produced the same effects in rainbow trout, common carp and African catfish sperm (Rurangwa et al. 1998).

Although this has been difficult to document, because most of the studies have been performed on wild fishery species and there are numerous compounds and effects on sperm, the possibility of establishing contaminated broodstocks in aquaculture is high. Farmed fish may also be exposed to heavy metals or estrogenic phytoestrogen contaminants in their diet, which could affect their sperm quality (Kime et al. 2001).

In some species such as the Senegalese Sole *Solea senegalensis* wild-captured breeders have been considered to be the best option for broodstock establishment and their sperm quality was considered better than that from stocks spawned in captivity (Cabrita et al. 2006). This fact was also attributed to a loss of genetic variability (Porta et al. 2006). However, in most species some degree of domestication and selection will increase the quality of breeders, and more control of sperm quality in future generations is expected. Currently, with the development of genetic tools for broodstock characterization and paternity identification, the idea that wild fish must be introduced regularly to avoid an undesired level of inbreeding could be progressively reduced. These tools together with appropriate management of reproductive stocks could avoid the problems encountered in the past with F_1, broodstock consanguinity.

The Importance of Understanding Male Maturation

One of the most important objectives of aquaculturists is to control the reproductive process of fish in captivity. Sperm maturation is crucial to ensure good fertility rates, and unfortunately, many fish exhibit reproductive dysfunctions when reared in captivity, producing poor quality sperm. In males these problems are usually related to low volumes and quality of milt. The fact that these fish are not exposed to natural conditions during the reproductive period can cause hormonal failures that produce the above-mentioned dysfunctions. In those species in

which milt must be obtained directly from the testes (e.g., silurid catfishes and sex-reversed salmonids) the problems could be even greater. In sex-reversed salmonids spermatozoa are non-motile, because they have not undergone several processes that take place in the seminal duct, and therefore have no fertilization potential. It is also well known that in many species, the sperm obtained from males in the reproductive season has better quality, in terms of fertilization rates, than do milt samples obtained out of season. In this section factors that affect sperm quality will be considered, paying attention to the physiological mechanisms that control fish sperm maturation and quality.

Period of Sperm Collection

The period for sperm collection is decisive to obtain mature sperm of high quality. Sperm motility and the total volume and density of expressible milt undergo seasonal changes in many species. These changes occur not only throughout the year but also within the reproductive season. It has been demonstrated that cryopreserved sperm obtained from the testis of sex-reversed trout in winter (the natural breeding season in this species) produces higher fertilization rates than do sperm samples obtained in spring (Robles et al. 2003).

Temperature and photoperiod are the two main factors changing between seasons and could affect sperm characteristics. It has been suggested that temperature changes might play a role in the mammalian fertilization process by altering membrane fluidity (Bell et al. 1997) and is likely to act in some fish in a similar way. It has been suggested that the temperature at which gametogenesis occurs could alter sperm quality (Labbé and Maise 1996). It was shown that rainbow trout which performed gametogenesis under a low temperature regime presented a higher content of cholesterol and phospholipids in the plasma membrane of spermatozoa than did fish held at higher temperatures. It was also noticed that this fact correlated with lower fertility rates in cryopreserved sperm. Photoperiod is the other factor that could affect sperm maturation and also changes depending on seasonality. However, it has been demonstrated that photoperiod manipulation did not affect the proportion of maturing Arctic char *Salvelinus alpinus* (Frantzen et al. 2004). Nevertheless, photoperiod plays an important role in the control of hormonal profiles and, as will be explained, hormonal levels affect sperm quality. In the forktail rabbitfish *Siganus argenteus* which spawns synchronously around the last quarter of the moon (when sperm motility peaks) a correlation between hormonal changes in the testis and lunar periodicity has been demonstrated (Rahman et al. 2003*)*. This study showed that the use of lunar cues in this species promotes modifications in the higher centers of the hypothalamus-pituitary-gonadal axis.

Changes in sperm quality are also observed within the reproductive season, and sperm motility is usually reduced at the end of the season. This reduction was attributed to the ageing of the spermatozoa and reduction in the steroidogenic-producing capacity of the testes (Nagahama 1994). On the other hand, changes in sperm density have been detected within the reproductive period. A reduced sperm density at the beginning of the spermiation period can be explained physiologically because it occurs in response to an increase in seminal fluid production controlled by the luteinizing hormone, which increases the intra-testicular pressure and enables the spermatozoa to be transported to the sperm ducts (Billard 1986). In some fish there is an increase in sperm density at the end of the spawning season accompanied by a decrease in the sperm volume. Nevertheless, such changes are not observed in all species. Substantial changes in sperm density were not observed in red porgy *Pagrus pagrus*, and high motility occurred at the end of the reproductive period (Mylonas et al. 2003). Some authors (Lahnsteiner et al. 2005) have found that linearity of movement of rainbow trout spermatozoa is lower at the beginning of

spawning, and consider this phenomenon as a sign of sperm immaturity in trout. However, this does not seem to be a good indicator in other species such as the European smelt *Osmerus eperlanus* (Kowalski et al. 2006).

Hormonal Treatments and Sperm Quality

Hormonal treatments have been successfully used since the 1930s in a variety of fish species to improve sperm characteristics or to overcome reproductive dysfunctions. Initially, gonadotropins from piscine or mammalian origin were employed, but since then it has become possible to induce native production of gonadotropin from the pituitary by employment of gonadotropin-releasing hormone agonists (GnRHa) (Zohar and Mylonas 2001). A newer generation of hormonal manipulation therapies has arisen with the synthetic GnRHa, which can be incorporated into slow delivery systems that release the molecule and induce increases in sperm production (Zohar and Mylonas 2001). This method provides a clear advantage in relation to administration by single injections, which do not stimulate long-term elevation of sperm production, and successive injections that can produce injures in the fish. In addition, single injections usually produce a transient elevation of seminal plasma production with a small increase in spermatozoa production, resulting in a lower spermatocrit (García 1991). Nevertheless, the induction of milt thinning by hormonal treatment is sometimes advisable. This is the case in some flatfish species in which sperm is viscous in captivity and cannot readily fertilize eggs (Vermeirssen et al. 1998, 2000).

As an example of these hormonal treatments, it has been observed in the European sea bass *D. labrax* that precocious males produce milt of comparable sperm characteristics to adult individuals and that treatment of precocious males with human chorionic gonadotropin (hCG) can enhance milt volume without affecting other sperm characteristics, including fertilizing ability (Schiavone et al. 2006). It has been demonstrated that GnRHa-delivery systems stimulate not only seminal fluid production but also the spermatozoa production in striped bass *Morone saxatilis* and white bass *Morone chrysops*, without affecting the density, motility or fertilization capacity of the sperm produced (Mylonas and Zohar 2001). In some species, such as the Senegalese sole, attempts to enhance milt production by using GnRHa implants have been unsuccessful (Agulleiro et al. 2006, Guzmán et al. 2007), and more complex hormonal therapies are required to ameliorate reproductive dysfunctions. In fact, GnRHa therapies are not successful when used in fish that are not at the final stages of gonadal development. It has been suggested that the pituitary must first be exposed to sex steroids before GnRHa can stimulate the release of gonadotropins (GtH). The combination of GnRHa with 11-ketoandrostenedione accelerated spermatogenesis and markedly increased sperm motility but the amount of milt produced after 28 d of treatment was reduced (Agulleiro et al. 2007). Recently, the inhibition of the dopaminergic system by pimozide combined with GnRHa treatment was reported to stimulate sperm production from this species (Guzman et al. 2007).

For many farmed fish, hormone supplementation is not always sufficient and the stimulation of physiological changes modifying environmental conditions, mainly the photo-thermal regime, is also required. Moreover, in some fish it is important that both sexes are kept together to ensure pheromone release. This is the case of the European smelt, in which injections of hCG were not effective for stimulation of semen production, but the maintenance of mixed sexes was found to increase sperm production and quality (Kowalski et al. 2006).

Testicular Sperm

In some species sperm maturation should be artificially induced *ex situ*. In sex-reversed trout that lack seminal ducts, the spermatozoa obtained from the testes are immotile. However, sperm can be incubated to artificially promote maturation. To promote sperm maturation, sperm samples obtained from the testes of sex-reversed trout were incubated for 2 hr in a commercial solution (MATURFISH®, IMV, France) at 4 ºC with oxygen supply (Robles et al. 2003). Different results in terms of fertilization rates were obtained using different methods of extracting sperm from the testes. Homogenization of the testis yielded poorer results than did extraction using incisions made with scalpel in the organ, because the latter method reduced contamination by blood cells and left immature cells in the testicular cysts.

Sex-reversed salmonids are not the only example in which sperm has to be obtained from the testis. In silurid catfishes, stripping of semen is almost impossible and hormonal treatments fail to induce semen release. For this reason, sperm extraction directly from the testes is the only available option. Studies suggest that the hormone oxytocin, used at an optimal concentration, may improve the fertilizing capacity of African catfish semen (Viveiros et al. 2003).

Male Improvement

Nutrition

As mentioned above, sperm quality is associated with male status and improvements of broodstock quality will have a direct effect on gametes. One way to achieve this goal is through nutrition, because several problems detected in sperm quality (in seminal plasma and spermatozoa constituents) have been identified as being caused by a deficiency in certain components in the diet. Spermatozoa plasma membrane phospholipids and cholesterol levels were associated with the type of food given to male broodstock. In Senegalese sole, males fed with mussels had more cholesterol in the spermatozoa plasma membrane than did spermatozoa from males fed with polychaetes (Cabrita et al. unpublished results). Also, in rainbow trout, sperm quality was improved using a phospholipid enriched diet that affected the phospholipid composition of the spermatozoa plasma membrane (Labbé et al. 1995, Pustowka et al. 2000). Supplementation with 0.4% arachidonic acid (ARA) also produced an increase of ARA levels in cod *Gadus morhua* sperm with a positive effect on sperm velocity. However, no effect was found in spermatocrit or in the percentage of motile cells (Bell et al. 2005). The same supplementation in halibut *Hippoglossus hippoglossus* produced a more stable spermatocorit during the reproductive season and extended the period of milt production. In European seabass, fatty acid diet enrichment increased the sperm counts and volume in males (Asturiano et al. 2001), and the same effect was found in Indian major carp *Catla catla* (Nandi et al. 2007). There is little doubt that polyunsaturated fatty acid improves sperm quality by increasing sperm production. It may also be possible that certain fatty acids could play a role in preventing lipid peroxidation in the presence of vitamins (E and C) (Gladine et al. 2007), increasing the resistance of plasma membrane to reactive oxygen species (ROS) generated during sperm ageing. More studies need to be conducted in this matter.

Similarly, other components introduced in the diet such as vitamins (E and C) can be incorporated in the composition of seminal plasma and play an important role in spermatozoa quality (Ciereszko and Dabrowski 1995, Lee and Dabrowski 2004), increasing sperm antioxidant resistance to ROS. Production of ROS in semen is involved in changes in sperm membrane fluidity, DNA fragmentation, protein damage, mitochondria impairment, and consequently, in a

decrease in motility and fertility (De Lamirande and Gagnon 1995, Sanocka and Kurpisz 2004). Recent studies on diet supplementation with vitamin E in Arctic char, demonstrated a reduction of lipid peroxidation in sperm (Mansour et al. 2006). Semen contains a powerful antioxidant defense system, but a balance of natural ROS production and antioxidant system must be maintained. This could probably be achieved through diet control and correct supplementation of fish meals and could have a direct effect on sperm quality.

Stress and Disease Resistance of Stocks

Environmental stressors are important factors that limit fish performance under aquaculture conditions, inducing several dysfunctions. In the particular case of fish broodstock, stress conditions can affect gamete quality. The response to stress in teleost fish is highly individualized, where some fish display a consistently high or low stress response. Thus, broodstock improvement in terms of selection to stress responsiveness may reduce the negative effects of stressors (Pottinger and Carrick 2000, Wang et al. 2004), collaborating in the maintenance of sperm quality. Studies have reported the relationship between stress responsiveness and reproductive performance in males associated with gamete quality. Two subpopulations of rainbow trout and striped bass broodstock males, were identified according to stress resistance (males with low stress responsiveness and males with high stress responsiveness) (Pottinger and Carrick 2000, Castranova et al. 2005). However, no significant differences in sperm motility and cell concentration were found between the subpopulations, although the levels of sex hormones were lower in high stressed males. Recently, their studies have reached the same conclusions and sperm quality in terms of cell concentration, production, motility, and frequency of milt production were not different between subpopulations in the Senegalese sole (Cabrita et al. 2008). Although selective breeding for stress tolerance is a strategy that has been employed in economically important animals such as turkeys (Brown and Nestor 1973), chickens (Gross and Siegel 1985) and other poultry (Freeman 1976) that could benefit aquaculture, the main effect on improvement of sperm quality needs more attention and should not be discarded.

Fish disease can also affect reproduction and subsequently gamete quality, limiting male reproductive success. The production of disease-resistant strains has been attempted by producing transgenic fish, however there is little information on their sperm quality. Thus we will focus the improvement of male sperm quality on a physiological and natural selection basis. Sperm cells are immunologically recognized as non-self in the male reproductive tract, and therefore, can be attacked by the immune system. To avoid this, testes have a haemato-testicular barrier avoiding contact between germinal cells and lymphocytes and males suppress their immune system during spermatogenesis. High levels of sexual hormones (associated with high sperm quality) may contribute to suppressing immune response (reviewed in Klein 2000), thus aiding in the production of high quality gametes. As a result, susceptibility to disease increases during the reproductive season and any stimulation of the immune system could negatively affect sperm production. It was found that the intensity of infection by a nematode species located outside the testes was negatively associated with sperm quality in infected male Arctic char sampled during their spawning period (Liljedal et al. 1999). This suggests that the extra-testicular immune environment may affect the production of high-quality sperm and that parasite infections located in the extra-testicular soma may reduce sperm quality.

The selection of high quality males could also be done on a natural selection basis by female cryptic choice, but in this case it is difficult to predict the results at a fish farm. Females

are able to detect the presence of individuals with high concentration of parasites and thus select mates according to male sperm quality (Kortet et al. 2004). A number of recent studies have investigated another possibility of female cryptic choice by selecting sperm on a genetic basis (Simmons et al. 1996, Stockley 1997). Genes from the major histocompatibility complex responsible for disease resistance and immunological recognition of self and non – self (Janeway 1993), were shown to be expressed in the sperm membrane surface of several vertebrate species with external fertilization (Bakker and Zbinden 2001, Landry et al. 2001, Ziegler et al. 2002, Skarstein et al. 2005), producing a differential response by the female or the egg to sperm from different male genotypes (Birkhead and Pizzari 2002). Thus, females could in a certain way, select and produce future breeders with more resistance to certain disease. However, this kind of selection cannot be easily controlled by fish farmers in most of the cultured species. Moreover, it is not clear that these mechanisms are present in all cultured fish species and, if they were, how they could be applied in fish maintained in captivity.

Husbandry Conditions

The maintenance of broodstock in appropriate conditions for each species is an important requirement for the production of high quality sperm. This is related to the physico-chemical aspects of water (temperature, oxygen, and salinity), photoperiod, type of tanks, and density of fish among other factors. In farmed species it is important to establish the appropriate husbandry conditions to avoid influences in gamete quality. These aspects have been reviewed in Rurangwa et al. (2004) and Cabrita et al. (2008).

Final Remarks

Management of fish reproduction with culture or conservation purposes requires the use of the best males as breeders. Quality of sperm can be assessed by analyzing different parameters with simple methods to very sophisticated approaches. Nevertheless, the identification of producers of good sperm is not always a guaranty of optimal reproductive performance, taking into account that many factors, from life history to social context or breeding conditions, can influence success. Knowledge of all factors influencing sperm quality can help to develop appropriate selection programs to improve quality of male broodstocks.

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New Chapter

Estimation of Fish Sperm Concentration by Use of Spectrophotometry

Rafael Cuevas-Uribe and Terrence R. Tiersch

Towards Harmonization: Some Lessons From Human Andrology Laboratories

A lack of standardization, wide variation among laboratories, and an urgent need for quality control led andrology laboratories worldwide to unify and developed standardized techniques and practices for semen analysis which were published by the World Health Organization (WHO) (Keel et al. 2002). The purpose of the WHO manual was to minimize variability among laboratories and to enable comparison of semen analysis results among laboratories. Standardized laboratory protocols and quality control are essential for meaningful comparisons of semen quality data from multiple sites (Brazil et al. 2004). Although the WHO manual is recognized globally as the "gold standard" for semen analysis, its acceptance has been less than universal. For example, most laboratories that participate in the German external quality control program for semen analysis do not use WHO-recommended methods (Cooper et al. 2007). Most inter-laboratory variation is attributed to the use of different techniques, and there is considerable disagreement regarding the relative accuracy and precision of various techniques (Brazil et al. 2004). Although improvements can be made in existing guidelines, protocols, and quality control systems, these current systems provide better tools than other non-standardized procedures (BjoRndahl et al. 2004).

New methods need to be validated for accuracy, repeatability, and precision before moving into standardization, which is a component of harmonization. To achieve harmonization, standards need to be developed for each method. For example, despite several efforts to standardize methods of semen analysis, sperm count is known to be subject to large inter-laboratory differences. Most variation is introduced through the use of different techniques (Jonckheere et al. 2005). Currently there is disparity in the equipment and procedural steps used for concentration measurements. This is because the standardization necessary for development of guidelines does not exist. After methods have been standardized, intercalibration comes into play. There is nothing inherently wrong in using different techniques as long as the results are accurate and consistent. Comparison by intercalibration is used to verify that values of a particular technique are correct. Thus the purpose of the intercalibration is not to harmonize the assessment method, but only the results (Buffagni and Furse 2006). Sometimes to ensure that the results of one technique are consistent with those of another, a transformation factor is applied to normalize the data (Poikane 2009). Criteria must be established to define the reliability of data for total allowable error specifications, and the extent of corrective measures that are acceptable. These criteria will define the procedures used for adjusting the data and compensation factors.

To minimize errors routine quality control needs to be established. Evaluation of an internal quality standard is essential to maintain accuracy, precision, and competence (Auger et al. 2000). Estimates of imprecision can be obtained from the internal quality control system. Imprecision can be reduced by regular training of personnel and by adopting best management practices. Workshops on standardization have been used to train andrology laboratory technicians (Toft et al. 2005). External quality control can also provide regular standardization

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checks and agreement among laboratories. External quality control programs should be directed at tangible elements (e.g., staff, instrumentation, equipment, and supplies) and at intangible elements (protocols and techniques) (Castilla et al. 2010). Adherence to the same standard procedures and criteria for each method will allow separate laboratories to work in unison. Harmonization results in making the outcomes comparable, not necessarily in making every laboratory do things in an identical fashion (van Nieuwerburgh et al. 2007). Harmonization allows choices between alternatives, out of which one or several can be adopted depending on the given circumstances (Figure 1).

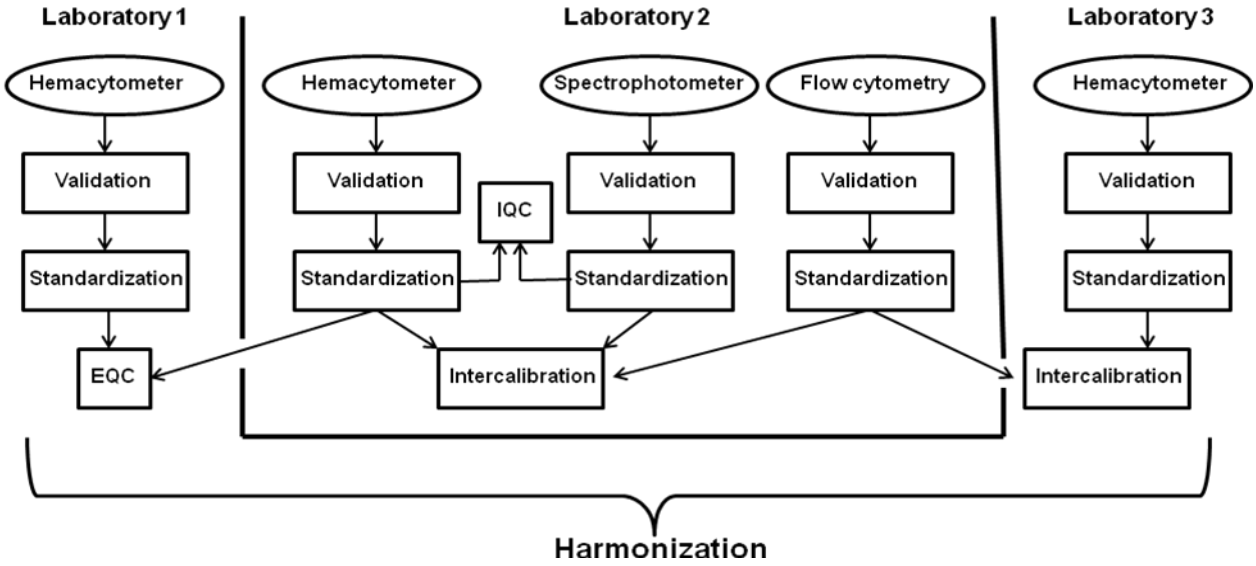

Figure 1. Schematic overview of a harmonization process (rectangles) for sperm counting methods (ovals) across three laboratories. Method validation is the first step to ensure that results are reliable. Standardization follows and allows development of guidelines for uniformity of response (a "top-down" approach). Many laboratories have more than one instrument that can perform the same function which enables internal quality control (IQC). IQC and external quality control (EQC) are needed to test variability within and among technicians, and among laboratories. Intercalibration seeks consistency in classification of results of different methods, and is used to determine if results from methods are accurate, credible, and comparable. Following intercalibration a method can be trusted and can move toward harmonization, the process of making different standards compatible and providing choices among methods. Harmonization bridges existing variation to provide a state of comparability, consistency, and similarity.

The Current Status for Sperm Quality Analysis in Aquatic Species

Currently, the single largest problem for sperm quality analysis (and standardization in general) in aquatic species is the lack of control and reporting of sperm concentration (Dong et al. 2007a). We place this problem foremost because sperm concentration will directly affect analysis results even for assays that are otherwise standardized (Tiersch et al. 2007). The first step in dealing with this problem is to recognize the importance in controlling and reporting sperm concentration, and to adopt and ultimately harmonize methods to estimate concentration. A variety of methods exist, each with advantages and disadvantages, and only a few of these have established methods. As stated above, after choosing a method, validation is necessary to move into standardization (Figure 1) which allows development of guidelines or standardized

protocols for each method. After standardization, the next step is comparison and intercalibration between and among laboratories to ensure that values are precise and accurate. Consistency, accuracy, and comparability of different methods are the keys for intercalibration (Poikane 2009). Harmonization can be addressed after the development of standardized protocols and establishment of an intercalibration process. At present, as stated above, there is a lack of standardization in the performance and reporting of sperm analyses in aquatic species. The study of sperm quality would greatly benefit from a standardization of analytical methods and tools (Fauvel et al. 2010). This would facilitate collaboration among laboratories with the aim to develop uniform (standardized) procedures and to derive similar accuracies through intercalibration exercises (Rosenthal et al. 2010) and eventual harmoniation. To facilitate making the first step in this process for aquatic species, the focus of this chapter will be on turbidimetric estimation of sperm concentration by use of spectrophotometry. This is not meant to suggest that this is the best or only technique available to measure sperm concentration, but it is widely accessible, has been widely applied for a variety of species and applications, and can serve as an overall representative model of the opportunities and problems inherent in other aspects of gamete quality analysis in aquatic species.

Principles of Turbidimetric Analysis

Whenever light strikes an object, the light can be scattered (reflected), absorbed, or passed through the object (refracted). The extent of light loss can be determined by measuring the amount scattered or reflected (nephelometry) or the amount of light transmitted (turbidimetry) (Csuros and Csuros 1999). In a turbidity measurement a spectrophotometer is used to measure the absorbance of light as a function of wavelength as it passes through a sample. The measurement of absolute absorbance depends in the separation of scattered light from the attenuation of light (absorbance). The sensitivity to measure light loss varies depending on the type, number, and position of detectors. For example, the sensitivity to measure absorbance increases if a detector is positioned far from the cuvette (sample container) (Figure 2). For this reason there are differences in the accuracy of various instruments. This is why several authors avoid use of the term "absorbance" and refer to spectrophotometric output as "apparent absorbance", "optical density", or "turbidity" (Poole and Kalnenieks 2000).

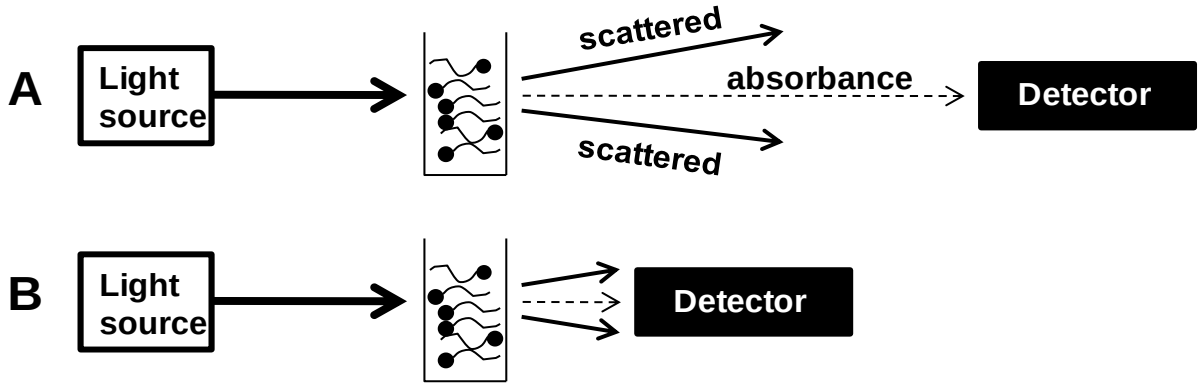

Figure 2. The measurement of absorbance varies depending on position of the detector. For accurate measurement it is necessary to eliminate or minimize the effect of light scattering. A) Positioning of the detector far from the sample enhances sensitivity because it will detect less scattered light. B) When the detector is close to the sample, scattered light will cause an artifactual reduction of absorbance (modified from Poole and Kalnenieks 2000).

Turbidity is routinely used as a measure of biomass concentration because the amount of light lost or scattered is inversely proportional to the cell concentration or directly proportional to the absorbance (Csuros and Csuros 1999). The apparent absorbance of a cell suspension depends on the wavelength used, the pathlength, and the cell concentration of the suspension. When nephelometry is used, a wavelength is chosen to optimize sensitivity and dynamic range, and to minimize the effects of light absorption by the cells or the medium components. By choosing a wavelength where no light absorption is recorded (e.g., 320-800 nm for proteins), the light striking the detector will be result only from light scattering. As a rule, choosing lower wavelengths will provide greater sensitivity of optical density measurements, but longer wavelengths will provide greater linearity over the same range of cell suspensions (Poole and Kalnenieks 2000). When turbidimetry is used, the wavelength selected is based on the maximal absorbance peak (e.g., 260 nm for nucleic acids). Sperm concentration is directly proportional to the absorbance and indirectly proportional to percentage transmittance (Csuros and Csuros 1999). To quantify an absorbance reading, a quantitative enumeration must be made (e.g. hemacytometer and coulter counter) to correlate with the apparent absorbance.

Estimation of Sperm Concentration in Livestock

The importance of reporting sperm concentration due to individual male variation and to allow comparison of studies was recognized early as being essential by the bull sperm industry. This industry next focused on development of rapid methods for accurate estimation of sperm concentration which continues today (Table 1). Different methods have been developed, ranging from comparison of sperm suspensions with opacity standards (Burbank 1935) to flow cytometric methods (Evenson et al. 1993). These methods vary in precision (Table 1).

Table 1. Coefficient of variation (repeatability) of different methods for estimation of sperm concentration reported in representative studies (arranged chronologically).

Animal studied	Hemacy-tometer	Sper-matocrit	Spectro-photometry	Coulter counter	Flow cytometry	Reference
Chicken	6.4	3.8	3.7	—	—	Taneja and Gowe 1961
Chicken	12.8	8.3	4.8	—	—	Taneja and Gowe 1961
Turkey	41.1	—	26.6	30.1	—	Brown et al. 1970
Chicken	17.9	9.9	2.2	1.6	—	Brillard and McDaniel 1985
Boar	12.3	—	2.9	2.3	—	Paulenz et al. 1995
Boar	7.1	—	10.4	—	2.7	Hansen et al. 2006
Bull	7.8	—	4.1	—	2.3	Prathalingam et al. 2006

The first record of using turbidity to measure sperm concentration in livestock dates back 70 yr (Comstock and Green 1939). The National Association of Animal Breeders has developed guidelines for measurement of sperm concentration by turbidity (Foote 1972, Foote et al. 1978). A survey among laboratories was used to identify the sources of variation including the spectrophotometer used, wavelength used, method of calibration, type of diluents, and dilution rate used (Foote et al. 1978). From the laboratories interviewed, 80% were using the same spectrophotometer and most of them were using the same wavelength (550 nm). It was concluded that the accuracy of estimating sperm concentration using spectrophotometer was

dependent on the calibration and standard curve used to correlate absorbance values with hemacytometer sperm counts.

The Importance of Estimating Sperm Concentration in Aquatic Species

The urgent need to standardize sperm studies has been emphasized recently in a workshop (Rosenthal 2008), symposium (10^{th} International Symposium on Spermatology 2006,Tiersch et al. 2007), and publications (Dong et al. 2005a, Dong et al. 2007a). Standardization is required to reproduce and optimize reports and protocols. A lack of standardization of sperm concentration has led to variability in results and reporting of studies (Dong et al. 2005a, Tiersch et al. 2007). Accurate estimation of concentration is necessary for a wide variety of topics including standardization of cryopreservation (Dong et al. 2007a), determination of optimal sperm-to-egg ratios (Suquet et al. 1995), calibration of ultraviolet irradiation to induce gynogenesis (Mims et al. 1997), assessment of spermiation following hormonal stimulation (Miranda et al. 2005), assessment of sperm production following sex manipulation (Fitzpatrick et al. 2005), nutritional studies (Rinchard et al. 2003), estimation of sperm motility (Cosson 2008), optimization of staining with fluorescent dyes (Paniagua-Chavez et al. 2006), and reproductive toxicology (Aravindakshan et al. 2004).

Uncontrolled variation in sperm concentration is one of the main reasons for the inconsistency observed among various studies associated with cryopreservation and fertilization (Dong et al. 2007a), and as such determination of concentration should be considered as an essential parameter in the assessment of sperm quality (Aas et al. 1991, Cabrita et al. 2009). Sperm volume and concentration vary among species (Piironen and Hyvarinen 1983) and individuals within the same species, with reported concentrations ranging between 2×10^6 to 6.5×10^{10} cells/ml (Leung and Jamieson 1991, Vuthiphandchai and Zohar 1999, Alavi et al. 2008). These differences are due to factors such as the stage of spawning season (Munkittrick and Moccia 1987), seasonal variation (Alavi et al. 2008), strain and genetic backgrounds (Scott and Baynes 1980, Tiersch 2001), diet (Ciereszko and Dabrowski 2000), physicochemical and social environment (Fitzpatrick and Liley 2008), disease (Rurangwa et al. 2004), and age (Poole and Dillane 1998).

The time, effort, and expense involved in rearing or capturing mature fish requires efficient use of sperm samples, especially for imperiled species (Tiersch et al. 1994). Common hatchery practices, if described in reports, typically include the addition of volumetric measures of gametes (e.g. 20 mL of sperm per 2 - 3 L of salmonid eggs; Willoughby 1999) without estimating the concentration or motility of the sperm (Aas et al. 1991). Commercial success of hatchery effort depends upon efficient utilization of available gametes (Erdahl and Graham 1987) and sperm can be in short supply due to limited numbers of broodstock or due to the small body sizes of aquarium fish used as biomedical models such as zebrafish *Danio rerio* (Tiersch 2001, Tan et al. 2010). In addition, the success or failure of cryopreservation protocols can be dictated by sperm concentration (Dong et al. 2007a). For these and other reasons it is therefore essential to routinely adopt a rapid, efficient, and accurate method for estimation of sperm concentration in aquatic species.

Methods Used to Estimate Sperm Concentration

There are several techniques used to estimate sperm concentration as part of the process to estimate sperm quality (Figure 3). Each technique has advantages and disadvantages, but no matter which technique is chosen it is essential to control, record, and report sperm concentration.

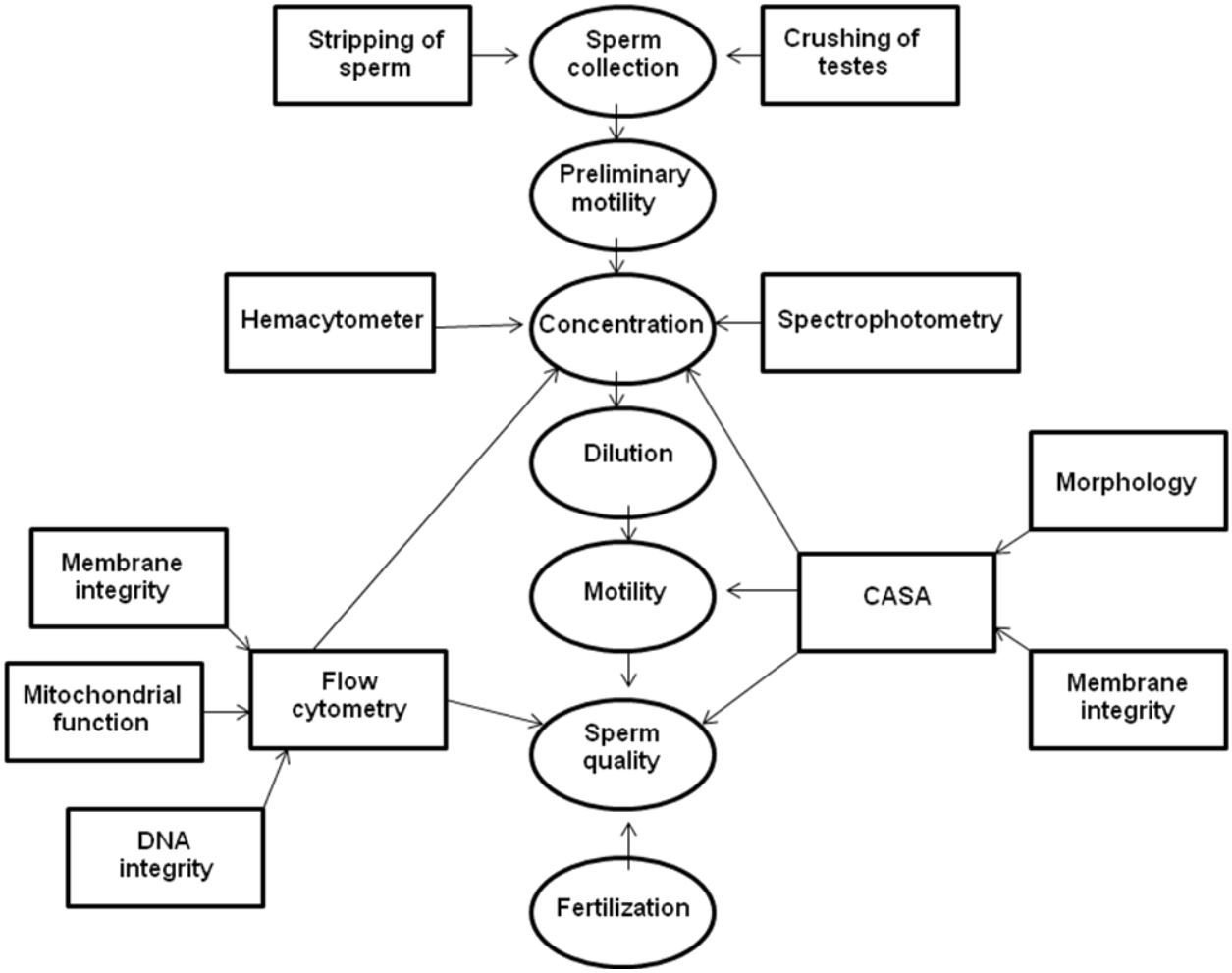

Figure 3. Relevant steps (ovals) for sperm quality assessments. Traditional sperm quality parameters (rectangles) have included motility, morphology, concentration, and membrane integrity (viability), with fertilization providing an ultimate assessment. More than one instrument can perform different tests. For example, concentration can be measured by use of computer-assisted sperm analysis (CASA), flow cytometry, hemacytometer, or spectrophotometry.

Fish sperm concentration has been assessed by three main techniques: counting in a hemacytometer chamber, estimation of spermatocrit, and turbidity evaluation (for more details see reviews by Rurangwa et al. 2004, Alavi et al. 2008, and Fauvel et al. 2010). Hemacytometer counting is precise and reliable for fish sperm, but is time consuming and thus cumbersome for fertilization protocols involving precise timing or many males (Fauvel et al. 1999, Alavi et al. 2008, Cabrita et al. 2009). Spermatocrit determination, although widely used, requires

centrifugation of the milt and only provides a relative measure expressed as the ratio of packed sperm volume to total volume of sample instead of the number of cells per mL. Because the handling time for samples should be minimized, establishment of a rapid and reliable method for sperm concentration estimation is required. Spectrophotometric determination of turbidity is an efficient and inexpensive method to estimate cell concentrations, given that an accurate initial calibration is established (Foote et al. 1978, Dong et al. 2005a). The advantages of using photometric measurement of sperm concentration in aquatic species has been known at least since 1949 (Rothschild 1950) and the application of this method to fish can be dated back at least to 1971 (Billard et al. 1971). Since then, the direct relationship between sperm concentration and absorbance has been established in approximately 41 species of fish (most within the past 10 yr). Despite or because of this diversity of use, the application of turbidity to estimate sperm concentration has not been collectively studied, and remains unstandardized and variable in methods and reporting. By reviewing the literature addressing use of spectrophotometer to measure sperm concentration in fish (described below) we found that the majority (65%) of the studies did not describe how they established the absorbance-concentration standard curve, and just one-third of the studies (35%) gave a description of the calibration curve equations, or other spectrophotometric measurement protocols.

From this review it appeared that the necessity of developing separate calibration curves for each species or population was a significant hindrance to wider utilization of this method to determine concentration. Therefore, based on a previous study done in livestock which compared sperm concentrations from bull, boar, and stallion as determined by the use of a single calibration curve (Rondeau and Rouleau 1981), we decided to evaluate the feasibility of using a single calibration to measure sperm concentration across a range of fish species.

Thus, this chapter addresses two goals, the first was to review the literature on previous estimations on fish sperm concentration by the use of spectrophotometer. With this review our objectives were to: (1) provide an overview of the different types of uses for spectrophotometric analyses, and (2) highlight the sources of variations in the technology. The second goal was to evaluate the general utility of turbidity in determining sperm concentration in fish species. The research objectives were: (1) wavelength identification for sperm concentration assessment, (2) development of standard curves for turbidity estimations in seven species, (3) validation of regression models in estimations, (4) determination of the relationship of standard curves across various species, and (5) evaluation of the effects of other cell types such as blood on turbidity measurement of sperm concentration. To our knowledge this is the first review of this topic area and the first report that demonstrates the feasibility of a general curve (instrument-specific) that can be used to measure concentration in fishes where sperm is collected by stripping, and with further procedural modification could apply to aquatic species in which the testes are crushed.

Materials and Methods

Goal One: Literature Review

We sought to use the literature review to form a database and to describe the previous uses and application of turbidity to estimate the sperm concentration in fishes. The first problem that we encountered was the difficulty of identifying scientific publications that used spectroscopy to measure sperm concentration. A simple search using the search terms “sperm concentration” and “fish spectrophotometer” in Google scholar (*scholar.google.com*) resulted in 296 publications. While compiling the publications that specifically used spectrophotometer to

estimate the sperm concentration in fish, two truly influential articles were identified (Suquet et al. 1992a, Ciereszko and Dabrowski 1993) that most of the studies using this technology made reference to. Based on the citations listed for these articles and using ISI Web of Knowledge℠, we found 52 citations for Suquet et al. (1992a) and 90 citations for Ciereszko and Dabrowski (1993). Overall between the yr 1971 and 2009 we found a total of 71 articles (in 32 journals), 9 meeting abstracts, and 2 dissertations that specified use of spectrophotometer to measure sperm concentration. The two journals publishing the most articles were *Aquaculture* (Elsevier: *www.elsevier.com)* with 13 articles and *Aquaculture Research* (Wiley-Blackwell: *www.wiley.com/WileyCDA/*) with 11 articles. The categories within the database constructed were: country of research (based on address of first author), species of fish, purpose of the study, description of the turbidity method, wavelengths tested for maximal absorbance, optimal wavelength used, absolute determination method for the generation of standard curves, correlations between methods, and concentration ranges tested.

Goal Two: General Curve Development

Sperm Collection

The scientific name, common name, sources of fish, and collection methods were listed for the seven species studied (Table 2). The studies were performed for tilapia during January to February of 2003 and the rest during March to August of 2004 at the Aquaculture Research Station of the Louisiana State University Agricultural Center in Baton Rouge.

Table 2. Scientific and common names (arranged by phylogenetic order), sources, sperm collection methods, and published work reporting other results obtained for the same fish.

Common name	Species	Source	Collection method	Dilution ratio[a]	Extender solution	Reference
Paddlefish	*Polyodon spathula*	Forest Hill, LA[b]	Stripped	2:1	HBSS200[c]	Mims and Shelton 2005
Blue catfish	*Ictalurus furcatus*	Inverness, MS[d]	Crushed testis	10:1	HBSS300	Lang et al. 2003
Channel catfish	*Ictalurus punctatus*	Baton Rouge, LA[e]	Crushed testis	10:1	HBSS300	Bates et al. 1996
			Sliced testis	none	HBSS300	Riley et al. 2004
Striped bass	*Morone saxatilis*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300[g]	Thirumala et al. 2006
White bass	*Morone chrysops*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300	Thirumala et al. 2006
Red drum	*Sciaenops ocellatus*	Gulf of Mexico, LA	Stripped	5:1	C-F HBSS200	Wayman et al. 1998
Tilapia[h]	*Oreochromis* sp.	Tiltech Aquafarm, LA	Stripped	32	HBSS300	Segovia et al. 2000

a sperm : extender
b Louisiana Department of Wildlife and Fisheries, Booker Fowler Fish Hatchery
c Hanks' balanced salt solution at 200 or 300 mOsmol/kg
d Harvest Select Farms
e Louisiana State University Agricultural Center, Aquaculture Research Station
f Kent Sea Tech, now Kent BioEnergy Corporation
g C-F HBSS: calcium-free HBSS
h Nile tilapia O. niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

The fish were anesthetized using tricaine methane sulfonate (MS-222, Western Chemical Inc., Ferndale, WA, USA) at 100-150 mg/l (Coyle et al. 2004) prior to stripping. They were removed from anesthesia and the genital papilla was dried with paper towels to avoid activation or contamination of the sperm by water. Samples were stripped carefully, to minimize contamination with urine or feces, and were diluted in Hanks' balanced salt solution (HBSS) or

calcium-free HBSS (C-F HBSS) of appropriate osmotic strength (Table 2). Because sperm cannot be stripped from ictalurid catfishes, testes were removed surgically and crushed in 300 mOsmol/Kg HBSS at a ratio of 1:10 (g testis:mL HBSS) to release sperm. This method yielded a mixture of cell types including mature and immature sperm cells, and somatic cells such as erythrocytes. Channel catfish testes were also sliced and squeezed to directly collect a relatively pure sample of sperm for research purposes.

Hemacytometer Counts and Dilution Preparation

An aliquot of each sperm sample was diluted before counts were made with a hemacytometer (Reichert bright-line, Hausser Scientific, Horsham, PA, USA). A 1:32 dilution (sperm:HBSS) was used for all species, except for red drum (1:500) and white bass (1:1000) which produce highly concentrated sperm samples (> 10^{10} cells per ml). Sperm concentrations were calculated using the average of four replicate hemacytometer counts with the following equation:

(Mean of quadruplet counts x dilution factor) x 50,000 = cells/ml

After the initial sperm concentrations were calculated, the solutions were diluted to contain 10^9, 10^8, 10^7 and 10^6 sperm cells/mL, and these concentrations were validated again by hemacytometer counts.

Spectrophotometer Readings

A Spectronic 20 Genesys™ (Thermo Spectronic, Rochester, NY, USA) was used to obtain the absorbance measurements except where otherwise stated. Disposable 1.5-mL polystyrene cuvets (Semimicro, Fisher Scientific, Pittsburg, PA, USA) with a 10-mm pathlength were used for each sample. Blanks were set using 1.5 mL of the extender used for each species. Diluted sperm samples (1.5 mL) from all seven species were measured at five wavelengths (400, 450, 500, 550 and 600 nm). To determine the wavelength of maximum absorbance, a sperm concentration of 2.5 x 10^8 cells/mL was used in all fishes except for red drum for which 1 x 10^9 cells/mL was used.

Effects of Other Cells Types

To evaluate the influence of blood cells on photometric measurements, we collected blood, sperm from crushed testes, and a relatively pure sperm sample collected by pipet from sliced testes from three channel catfish. Three different concentrations were used (1 x 10^8, 1 x 10^7, and 1 x 10^6 sperm cells/mL) for the sperm samples collected directly from the testes. Blood was collected by caudal puncture using sodium heparin as an anticoagulant (from Becton Dickinson Vacutainer™, Franklin Lakes, NJ, USA). In each of these sperm concentrations, whole blood (on the order of 10^9 blood cells per mL) was added to yield five different final volumetric proportions of blood and sperm (0.125%, 0.25%, 0.375%, 0.5%, and 1%). For example, to prepare one ml of sperm sample with 1% of blood, 10 µL of blood was added to 990 µL of sperm sample. The absorbance of these samples was measured using a spectrophotometer (Spectronic 20) at five wavelengths (400, 450, 500, 550 and 600 nm). The absorbance values were measured by using a scanning microspectrophotometer (Nanodrop® ND-1000 Wilmington, DE, USA) across wavelengths from 220 to 748 nm at 2-nm intervals.

Statistical Analysis

Simple linear regression was used for testing the correlation between absorbance and sperm concentration for each species at the five wavelengths. Data for sperm concentrations were logarithmically transformed (natural logarithm) prior to regression analysis (Berman et al. 1996). To test for significant differences ($P < 0.05$) among linear models, multisource regression with analysis of covariance was used. To test for correlation among species, and among blood percentages, multiple regression was used. Because R^2 values increase with the addition of new variables, the adjusted R^2 (Neter et al. 1996) was used to compensate for added explanatory variables. After determining that there were no differences among them ($P >0.05$), the observations from Nile tilapia, blue tilapia, Mississippi commercial strain and Florida red tilapia were pooled to strengthen the sampling for tilapia species. The software used for all analyses was SAS® 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Literature Review:

Overview of Uses for Turbidity Analyses

The 82 publications collected represented 18 countries. This reflected a wide diversity of research types and a wide variety of study purposes utilizing spectrophotometer (Table 3). Estimations of sperm concentration by turbidity have been established in at least 41 species of fish and 3 species of mollusks. About 60% of the studies were done in the past 10 yr.

Sources of Variation in the Reports

Approximately 65% of the publications did not report how they standardized the technique, which included the wavelengths tested, wavelengths selected, and sperm concentration ranges tested (Table 3). Wavelengths between 260 and 660 nm have been used to determine sperm concentrations in fish. The most frequently used (20%) wavelength was 505 nm (Figure 4). The most cited reference of technique (50%) was by Ciereszko and Dabrowski (1993), and Ciereszko was an author for 25% of these publications. Another source of variation was the type of spectrophotometer used, as less than half (31 of 75) of the studies named the model of the spectrophotometer. Of these, total of 17 different models were reported.

Establishment of General Curve:

Wavelength Identification

The absorbance spectra of sperm from the seven species were stable within the wavelengths tested (Figure 5); there were no absorption peaks or discrete wavelengths of maximum absorbance within the range tested (400 – 600 nm). Therefore, any visible wavelength could be appropriate for use to generate a standard curve.

Table 3 Previous studies that estimated sperm concentration of aquatic species by spectrophotometry (presented in chronological order). Standarization of the technique include: wavelength tested, absolute determination, coefficient of determination, and concentration range of the standard curve.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Rainbow trout	*Oncorhynchus mykiss*	Spermatogenesis	Yes	200 - 600	410	hemacytometer	0.99	1×10^{10} to 2.8×10^{10}	Billard et al. 1971
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	ND*	ND	410	ND	ND	ND	Billard 1974
Common carp	*Cyprinus carpio*	Spermiation	ND	ND	410	ND	ND	ND	Takashima et al. 1984
Turbot	*Psetta maxima*	Sperm concentration	Yes	300 - 750	420	hemacytometer	0.94	5×10^{9} to 8×10^{10}	Suquet et al. 1992a
Turbot	*Psetta maxima*	Spermiation	ND	ND	420	ND	ND	ND	Suquet et al. 1992b
Rainbow trout	*Oncorhynchus mykiss*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.95	1.9×10^{9} to 2.1×10^{10}	Ciereszko & Dabrowski 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.71	3.4×10^{9} to 1.4×10^{10}	Ciereszko & Dabrowski 1993
Yellow perch	*Perca flavescens*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.94	3.7×10^{10} to 4.7×10^{10}	Ciereszko & Dabrowski 1993
Turbot	*Psetta maxima*	Sperm characterization	ND	ND	420	ND	ND	ND	Suquet et al. 1993
Eastern oyster	*Crassostrea virginica*	Fertilization trials	ND	ND	650	hemacytometer	ND	ND	Gaffney et al. 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm quality	ND	ND	ND	ND	ND	ND	Ciereszko & Dabrowski 1995
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1995
Turbot	*Psetta maxima*	Fertilization trials	ND	ND	420	ND	ND	ND	Suquet et al. 1995
Bleak	*Alburnus alburnus*	Sperm physiology	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996a
Blue mussel	*Mytilus edulis*	Larval culture	Yes	200 - 800	320	hemacytometer	0.98	1.3×10^{9} to 1.3×10^{10}	Del Rio-Portilla 1996
Yellowtail flounder	*Limanda ferruginea*	Sperm physiology	Yes	300 - 900	420	hemacytometer	0.92	2.7×10^{9} to 2.7×10^{10}	Clearwater 1996
Muskellunge	*Esox masquinongy*	Sperm characterization	Yes	400 - 800	610	hemacytometer spermatocrit	0.79	7.5×10^{9} to 3×10^{10}	Lin et al. 1996a
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	610	ND	ND	ND	Lin et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	Yes	ND	505	hemacytometer	ND	ND	Conget et al. 1996
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Reproductive performance	ND	ND	ND	ND	ND	ND	Dabrowski & Ciereszko 1996
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims & Shelton 1998
Common carp	*Cyprinus carpio*	Gynogenesis	Yes	ND	360	hemacytometer	0.78	$21.2 \pm 12.8 \times 10^{6}$	Porter 1998
Bluegill	*Lepomis macrochirus*	Gynogenesis	Yes	ND	360	hemacytometer	0.84	$6.9 \pm 2.7 \times 10^{6}$	Porter 1998
Brown trout	*Salmo trutta*	Sperm concentration	Yes	ND	505	hemacytometer	0.94	2.2×10^{9} to 2.7×10^{10}	Poole & Dillance 1998
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1998

Table 3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Atlantic salmon	*Salmo salar*	Reproductive performance	ND	ND	ND	ND	ND	ND	Estay et al. 1999
Bream	*Abramis brama*	Cryopreservation	Yes	ND	530	hemacytometer	0.97	6×10^9 to 2.1×10^{10}	Glogowski et al. 1999
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 1999
European bass	*Dicentrarchus labrax*	Fertilization trials	Yes	200 - 500	260	hemacytometer	0.97	4×10^7 to 2×10^8	Fauvel et al. 1999
Paddlefish	*Polyodon spathula*	Spermiation	Yes	ND	450	hemacytometer	0.86	1×10^8 to 1.6×10^9	Linhart et al. 2000
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	ND	ND	ND	Glogowski et al. 2000
Common carp	*Cyprinus carpio*	Sperm characterization	Yes	ND	610	hemacytometer	ND	$1\text{-}2 \times 10^5$	Dzuba et al. 2001
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	Yes	ND	600	hemacytometer	ND	ND	Miranda et al. 2001
Zebra mussel	*Dreissena polymorpha*	Sperm characterization	Yes	ND	500	hemacytometer	0.98	2.2×10^6 to 1.8×10^7	Ciereszko et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.69	5×10^8 to 9×10^9	Viveiros et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.88	ND	Viveiros et al. 2002
Sterlet	*Acipenser ruthenus*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Fertilization trials	ND	ND	ND	ND	ND	ND	Glogowski et al. 2002
Rainbow trout	*Oncorhynchus mykiss*	Gamete and embryo storage	ND	ND	ND	ND	ND	ND	Babiak & Dabrowski 2003
African catfish	*Clarias gariepinus*	Spermiation	Yes	ND	505	hemacytometer	0.85	2×10^8 to 1.2×10^{10}	Viveiros et al. 2003
African catfish	*Clarias gariepinus*	Sperm characterization	ND	ND	650	hemacytometer	ND	3×10^9 to 9×10^9	Mansour et al. 2004
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2004
Siberian sturgeon	*Acipenser baerii*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Sterlet	*Acipenser ruthenus*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Atlantic cod	*Gadus morhua*	Sperm quality	Yes	ND	260	hemacytometer	0.99	1×10^9 to 5×10^{10}	Suquet et al. 2005
Blue catfish	*Ictalurus furcatus*	Sperm concentration	Yes	400 - 600	600	hemacytometer	0.77	1.1×10^7 to 1×10^8	Campbell et al. 2005a
Channel catfish	*Ictalurus punctatus*	Sperm concentration	Yes	400 - 600	500	hemacytometer	0.53	1×10^6 to 3×10^8	Campbell et al. 2005b
Zebrafish	*Danio rerio*	Cryopreservation	Yes	460, 560, 660	All	hemacytometer	0.97	ND	Yang & Tiersch 2005
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	ND	ND	410	ND	ND	ND	Miranda et al. 2005
Paddlefish	*Polyodon spathula*	Aquaculture	ND	ND	450	hemacytometer	ND	2×10^8 to 1.7×10^9	Mims & Shelton 2005
Brown trout	*Salmo trutta* Linnaeus	Toxicology	ND	ND	405	hemacytometer	ND	ND	Lahnsteiner et al. 2005a
Rainbow trout	*Oncorhynchus mykiss*	Toxicology	ND	ND	ND	ND	ND	ND	Lahnsteiner et al. 2005b
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2005b
Pacific oyster	*Crassostrea gigas*	Sperm concentration	Yes	380 - 780	550, 581	hemacytometer	0.99	2×10^7 to 2×10^9	Dong et al. 2005a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005b
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005c
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2006
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	ND	ND	ND	Krol et al. 2006
European bass	*Dicentrarchus labrax*	Spermiation	ND	ND	260	ND	ND	ND	Schiavone et al. 2006

Table 3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2007a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	ND	ND	ND	ND	Dong et al. 2007b
Caspian brown trout	*Salmo trutta caspius*	Sperm concentration	Yes	ND	480	hemacytometer spermatocrit	0.91	7×10^8 to 6.5×10^9	Hatef et al. 2007
Brown trout	*Salmo trutta*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Wojtczak et al. 2007
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007b
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	Yes	ND	410	spermatocrit	0.98	ND	Fitzpatrick & Liley 2008
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Tuset et al. 2008
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2008
Arctic char	*Salvelinus alpinus*	Fertilization trials	ND	ND	ND	ND	ND	ND	Mansour et al. 2008a
Arctic char	*Salvelinus alpinus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Mansour et al. 2008b
Blue mussel	*Mytilus edulis*	Sperm concentration	Yes	200 - 800	320	hemacytometer coulter counter	0.99	9.4×10^5 to 1.1×10^7	Del Rio Portilla & Beaumont 2008
Powan	*Coregonus lavaretus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2008
Brook trout	*Salvelinus fontinalis*	Sperm concentration	ND	ND	ND	NucleoCounter	0.96	8.2×10^9 to 1.8×10^{10}	Nynca & Ciereszko 2009
Zebrafish	*Danio rerio*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.92	2.2×10^7 to 5.9×10^8	Tan et al. 2010
Swordtail	*Xiphophorus helleri*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.94	ND	Tan et al. 2010
Medaka	*Oryzias latipes*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.93	ND	Tan et al. 2010
Powan	*Coregonus lavaretus*	Sperm characterization	ND	ND	530	ND	ND	ND	Hliwa et al. 2010
European bass	*Dicentrarchus labrax*	Sperm quality	ND	200 - 500	260	hemacytometer	0.97	ND	Fauvel et al. 2010
Gilthead bream	*Sparus aurata*	Sperm quality	ND	200 - 500	260	hemacytometer	0.96	ND	Fauvel et al. 2010
Turbot	*Psetta maxima*	Sperm quality	ND	200 - 500	260	hemacytometer	0.95	ND	Fauvel et al. 2010
Wreckfish	*Polyprion americanus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.98	ND	Fauvel et al. 2010
Bluefin tuna	*Thunnus thynnus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.84	ND	Fauvel et al. 2010
Striped catfish	*Pangasianodon hypophthalmus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.86	ND	Fauvel et al. 2010
Atlantic cod	*Gadus morhua*	Sperm quality	ND	200 - 500	260	hemacytometer	0.99	1×10^6 to 5×10^7	Fauvel et al. 2010

*ND: not described; **Scaphirhynchus platorynchus

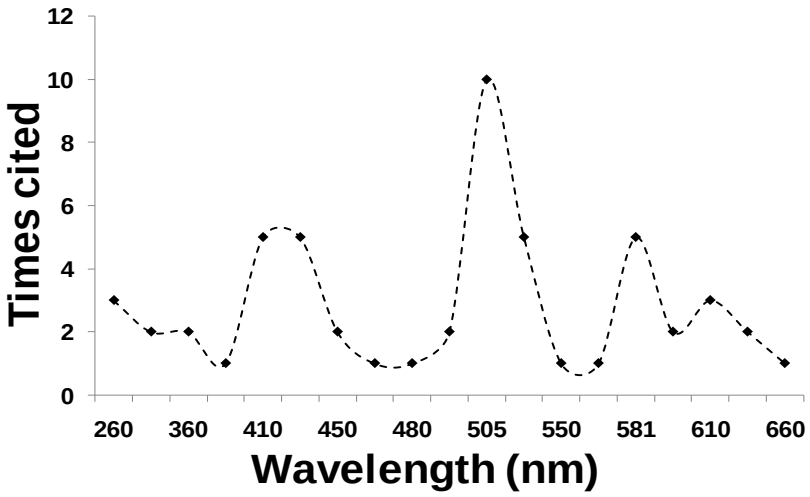

Figure 4. Frequency of use of various wavelengths evaluated in 54 previous studies for the development of standard curves to estimate sperm concentration in fishes.

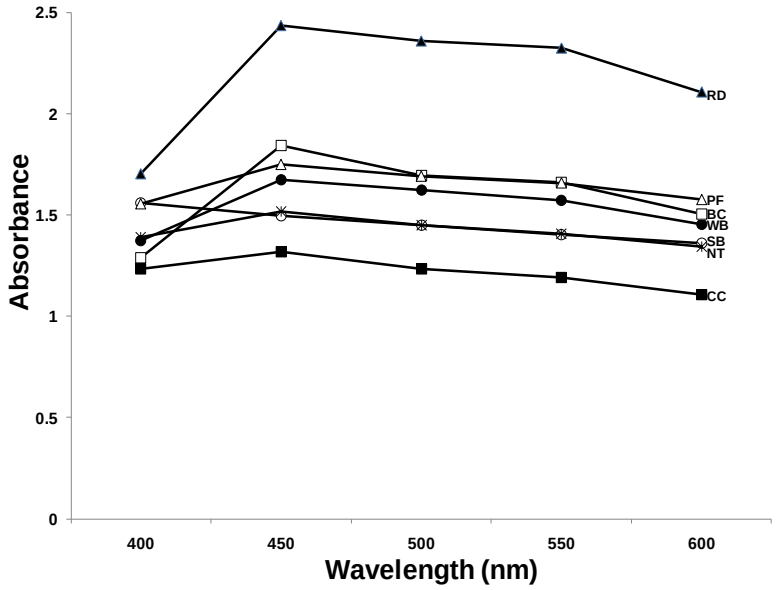

Figure 5. Absorbance spectra measured in this study for sperm of red drum (RD: filled triangles), paddlefish (PF: open triangles), blue catfish (BC: open squares), channel catfish (CC: filled squares), white bass (WB: filled circles), striped bass (SB: open circles), and Nile tilapia (NT: asterisks). Absorbance values were based on using 2.5 x 10^8 cells/mL for all species, except red drum at 1 x 10^9 cells/mL.

Development of Standard Curves

A linear relationship (R^2 values from 0.586 to 0.904) was found between the natural logarithm of sperm concentration (1 x 10^6 to 6 x 10^{10} cells/mL) assessed by hemacytometer counting and the corresponding absorbance in the different wavelengths used (Table 4). The strongest correlations at the different wavelengths remained stable for the majority of the species,

except at 400 nm for red drum (adjusted $R^2 = 0.040$). This indicated that there was a wide range of wavelengths that could be used to estimate sperm concentration.

Table 4. Standard curves, linear regression equations, and coefficient of determination from the logarithmic regression of sperm counts and absorbance at the different wavelengths tested.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue catfish	15	72	y= -2.994 + 0.215 lnX Adj R^2 = 0.810	y= -3.418 + 0.243 lnX Adj R^2 = 0.739	y= -3.130 + 0.222 lnX Adj R^2 = 0.730	y= -2.977 + 0.212 lnX Adj R^2 = 0.694	y= -2.678 + 0.190 lnX Adj R^2 = 0.703
Channel catfish	47	202	y= -2.067 + 0.162 lnX Adj R^2 = 0.587	y= -2.450 + 0.185 lnX Adj R^2 = 0.601	y= -2.348 + 0.176 lnX Adj R^2 = 0.603	y= -2.233 + 0.167 lnX Adj R^2 = 0.586	y= -2.118 + 0.158 lnX Adj R^2 = 0.592
Paddlefish	4	11	y= -6.799 + 0.446 lnX Adj R^2 = 0.901	y= -8.663 + 0.553 lnX Adj R^2 = 0.889	y= -8.567 + 0.544 lnX Adj R^2 = 0.885	y= -8.442 + 0.535 lnX Adj R^2 = 0.881	y= -8.106 + 0.513 lnX Adj R^2 = 0.877
Red drum	5	14	y= 1.495 + 0.010 lnX Adj R^2 = 0.040	y= -2.702 + 0.243 lnX Adj R^2 = 0.777	y= -3.246 + 0.265 lnX Adj R^2 = 0.805	y= -3.040 + 0.253 lnX Adj R^2 = 0.784	y= -1.228 + 0.157 lnX Adj R^2 = 0.791
Striped bass	12	45	y= -5.626 + 0.349 lnX Adj R^2 = 0.850	y= -6.457 + 0.397 lnX Adj R^2 = 0.860	y= -6.285 + 0.386 lnX Adj R^2 = 0.853	y= -6.129 + 0.376 lnX Adj R^2 = 0.846	y= -5.914 + 0.362 lnX Adj R^2 = 0.840
Tilapia[3]	69	114	y= -5.207 + 0.336 lnX Adj R^2 = 0.843	y= -6.523 + 0.410 lnX Adj R^2 = 0.820	y= -6.379 + 0.400 lnX Adj R^2 = 0.816	y= -6.214 + 0.389 lnX Adj R^2 = 0.812	y= -5.895 + 0.369 lnX Adj R^2 = 0.814
White bass	21	75	y= -4.476 + 0.289 lnX Adj R^2 = 0.871	y= -6.588 + 0.411 lnX Adj R^2 = 0.904	y= -6.525 + 0.406 lnX Adj R^2 = 0.902	y= -6.476 + 0.402 lnX Adj R^2 = 0.901	y= -5.879 + 0.366 lnX Adj R^2 = 0.903

1N = number of fish
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia Oreochromis niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

Validation of Regression Models

When validating the equation generated with the concentrations provided by hemacytometer counts, there were no significant differences between the observed values by counts and the predicted values of the standard curves, except when comparing the predicted concentrations from the general curve of all species (combining the data for crushed testis and stripped sperm) ($P = 0.001$).

Relationship of Standard Curves Among Species

When comparing the regression curves across species within individual genera (i.e. *Ictalurus, Morone,* and *Oreochromis),* there were no differences, except for blue and channel catfish at 400 and 450 nm, and white and striped bass at 400 nm (Table 5). When comparing the curves across all species (independent of the genus), there were significant differences for all the wavelengths ($P < 0.001$). However, when catfishes were removed from the model, there was no difference in the curves of paddlefish, red drum, striped bass, and tilapias for all the wavelengths except 400 nm (Table 5). Overall, the linear relationship of catfishes followed a pattern different from the values of the other species studied (Figure 6). When the absorbance values of catfishes were compared with those for other fishes within the absorbance range of 0.1 to 2.5 at concentrations of lower than 1×10^9 cells/mL, there was an overestimation of absorbance for the catfishes at any given concentration. This difference was likely because suspensions from crushed testes were contaminated with somatic cells such as erythrocytes which increased the

absorbance values. Overestimation was greater at lower sperm concentrations due to the higher relative proportion of somatic cells in relation to sperm cells (Figure 6).

Table 5. Comparison of standard curves among species. The curves were compared by an analysis of covariance (ANCOVA) at each wavelenght. Multiple regression was used to correlate among the species the absorbance, and natural logarithmic of sperm concentration.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue & channel catfish	2	274	p-value = 0.022	p-value = 0.028	p-value = 0.061	p-value = 0.068	p-value = 0.149
White & striped bass	2	120	p-value = 0.019	p-value = 0.633	p-value = 0.497	p-value = 0.379	p-value = 0.889
Tilapia[3]	4	114	p-value = 0.057	p-value = 0.296	p-value = 0.252	p-value = 0.248	p-value = 0.239
All species	8	533	R^2 = 0.774 p-value = <.001	R^2 = 0.806 p-value = <.001	R^2 = 0.811 p-value = <.001	R^2 = 0.805 p-value = <.001	R^2 = 0.805 p-value = <.001
All species without catfish	6	259	R^2 = 0.875 p-value = <.001	R^2 = 0.899 p-value = 0.358	R^2 = 0.896 p-value = 0.401	R^2 = 0.895 p-value = 0.334	R^2 = 0.890 p-value = 0.183
All species without blood	8	282	R^2 = 0.879 p-value = <.001	Adj R^2 = 0.899 p-value = 0.079	Adj R^2 = 0.896 p-value = 0.041	Adj R^2 = 0.894 p-value = 0.023	Adj R^2 = 0.900 p-value = 0.013
Blood	1	108	p-value = <.001	p-value = 0.208	p-value = 0.067	p-value = 0.188	p-value = 0.149

1N = number of species
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia O. niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

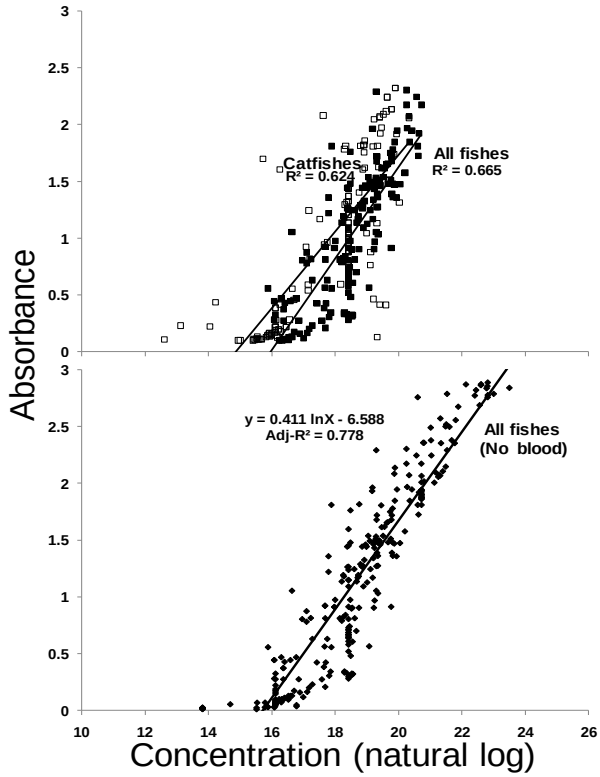

Figure 6. Relationship between absorbance at 450 nm and sperm counts by hemacytometer. Upper panel: regression lines for all fishes without catfishes, and catfishes only. The absorbance range was 0.1 to 2.5, and the maximum sperm concentration was 1 x 10^9 cells/ml. Lower panel: standard curve for all fishes, including catfish, with no blood.

Subsequent plotting of data for catfish sperm collected without blood (relatively pure samples) were combined with the data for paddlefish, red drum, tilapia, white bass and striped bass, and there was no significant difference for each wavelength except for 400 nm (Table 5). Also, the estimated values from the standard curve of all fish samples without blood had no significant differences (P = 0.181 at 450 nm) when compared with the hemacytometer counts. Moreover, plotting of the observed values of all species without blood against the standard curve generated from readings at 450 nm indicated a strong relationship (R^2 = 0.778) (Figure 6).

Effect of Blood

The interaction of the different blood percentages was significant for the 400 nm wavelength (Table 5). As the volumetric proportions of blood increased, the absorbance values also increased (Figure 7).

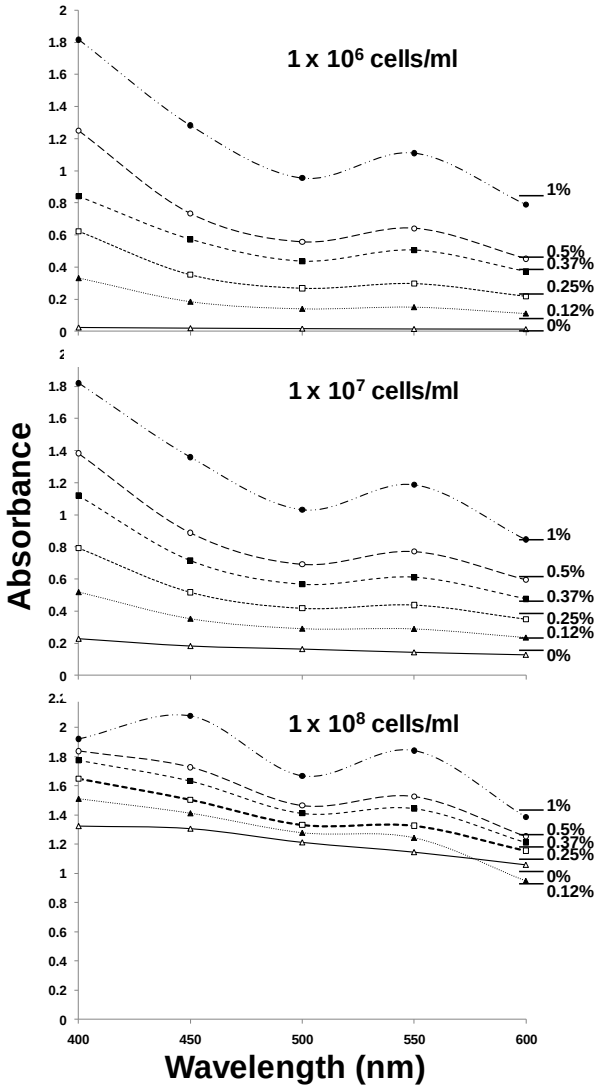

Figure 7. Absorbance values measured at wavelengths between 400 nm and 600 nm for three concentrations of channel catfish sperm with different volumetric proportions of blood. Upper panel: absorbance values using 1 x 10^6 sperm cells/mL with different percentages of blood; middle panel: 1 x 10^7 cells/mL; lower panel: 1 x 10^8 cells/mL.

A broad absorbance peak did not occur at low volumetric proportions of blood but the peak increased with higher proportions of blood. This is consistent with the absorbance spectra for pure blood samples. When the absorbance of blood was measured at different wavelengths, there was an increase in absorbance at 450 nm, with a maximum at 500 to 550 nm (Figure 8).

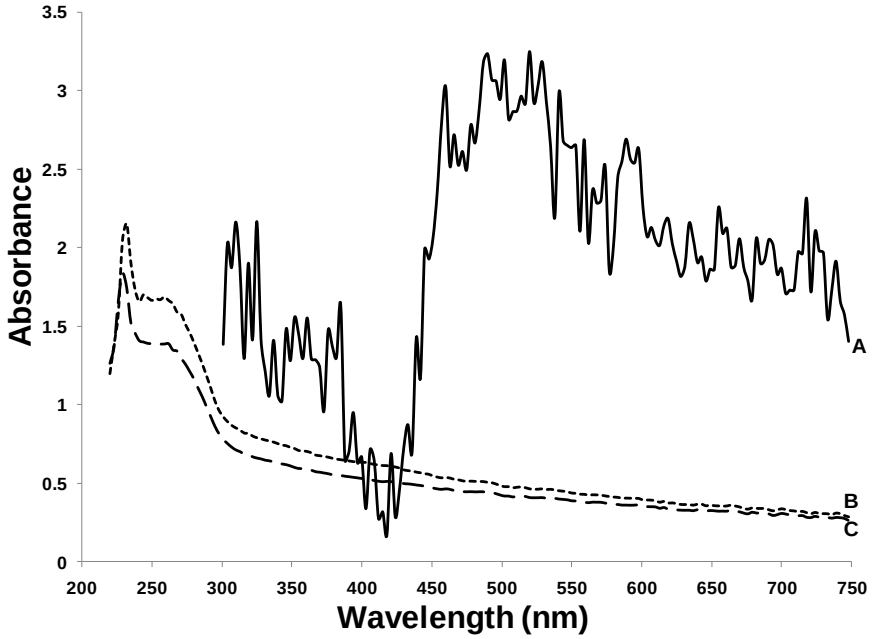

Figure 8. Absorbance values measured at wavelengths between 220 nm to 748 nm for: A) channel catfish blood, B) sperm collected from crushed testis, and C) sperm without blood. Absorbance values were the average of three males with a sperm concentration of 1 x 10^8 cells/mL. There was no maximal absorbance peak within the visible spectrum for the samples. The maximal absorption of blood was between 500 to 550 nm. Absorbance values of samples below 300 nm were variable.

Absorbance values of blood at wavelengths lower than 300 nm were inconsistent. However, the absorbance of sperm from crushed testes and sperm without blood remained constant. Although a maximum absorbance was detected at 230 nm, the absorbance values (greater than 1.2) yielded high variability (Figure 8). Within the visible wavelengths (390 – 750 nm) there was no absorbance peak. In general there was a gradual linear reduction in absorbance as the wavelength increased. Therefore, at low sperm concentrations (< 1 x 10^6 cells/mL) the absorbance values were influenced more by the presence of blood. As the sperm concentrations increased (> 1 x 10^7 cells/mL), there was an increase of absorbance caused by the sperm. When no blood was added the absorbance values were directly related to the sperm concentration (Figure 7).

Discussion

Sperm concentration is rarely reported during studies of aquatic species, and typically when reported does not include a description of the methods used. This leads to problems for comparison and reproducibility of published studies especially for activities such as cryopreservation and fertilization trials. The estimation of sperm concentration is essential for a range of activities including standardization of cryopreservation, optimization of fertilization, calibration of ultraviolet irradiation to induce gynogenesis, and study of spermiation following

hormonal stimulation. Despite measurements in more than 40 species, currently there is a lack of specific information regarding sperm concentration determination and how it relates to cryopreservation and fertilization in essentially all aquatic species (Tiersch et al. 2007). Traditionally in livestock species, sperm concentrations have been estimated by the use of cell counting devices such as the hemacytometer or other specialized counting chambers (Foote et al. 1978, Prathalingam et al. 2006). Although by observing the sperm using a microscope, other parameters such as morphology could be determined. But counting chambers are time consuming, and require the use of microscopes and trained technicians typically not available in hatchery settings. As such, most aquaculture work does not include sperm concentrations (Campbell et al. 2005a, Dong et al. 2005a).

In the search for faster and more practical ways to estimate relative or absolute sperm concentration, centrifugation (to determinate spermatocrit) and spectrophotometer (to determine turbidity) have been used. Because spermatocrit and absorbance are easy to measure, the choice of methods has generally been based on access to equipment (Tvedt et al. 2001). Spermatocrit is an indirect method which is expressed as the volume of sperm in relation to the total volume of sample (packed cell volume divided by total sample volume). One of the common problems in spermatocrit estimation is the lack of a clear separation between the packed sperm cells and the seminal fluid; this can lead to false estimations of spermatocrit. To avoid this problem, prolonged centrifugation times are needed, usually more than 10 min, but as long as 45 min in species with dense sperm samples such as striped bass (Vuthiphandchai and Zohar 1999). An additional problem is the relatively large volume (at least 0.1 ml) needed (Lin et al. 1996b).

Sperm evaluation should be rapid and effective so that samples can be processed efficiently to preserve initial quality and fertility (Foote 1980). Spectrophotometric determination of turbidity is recognized as a reliable, efficient, and rapid technique to estimate the concentration within semen samples in farm animals (Brillard and McDaniel 1985). From the previous work that used spectrophotometer to determine sperm concentration in aquatic species, there has been no attempt to evaluate the feasibility of generating a general calibration curve.

Literature Review:

Previous studies that estimated sperm concentration by spectrophotometry in fishes are characterized by a lack of description of the methodology used (Table 3). This failure of reporting and in defining procedures limits reproducibility, weakens results, and makes direct comparisons among studies problematic or impossible. There is a pressing need for development of standardized protocols. Less than 20% of the studies in our literature review tested different wavelengths to identify the wavelength of maximum absorbance (this may or may not be a large problem depending on the instrument used). Our review also found that one third of the studies used wavelengths between 500 to 550 nm which can be affected by the presence of blood in the samples (Figure 7). The reference cited most (18 of 54, 33%) in the methods sections of published papers was by Ciereszko and Dabrowski (1993). This publication addressed comparison of three methods for sperm concentration determination (i.e., spectrophotomer, hemacytometer, and spermatocrit). Almost all of these publications (16 of 18) did not state which of these three methods were actually used (e.g., Rinchard et al. 2001, Kowalski et al. 2006). In an attempt to compare previous studies with the present study, we estimated the sperm concentration from the equations described in six studies and plotted them (Figure 9). The differences among studies can be explained by the difference in instruments (see below) and the ranges of sperm concentration tested.

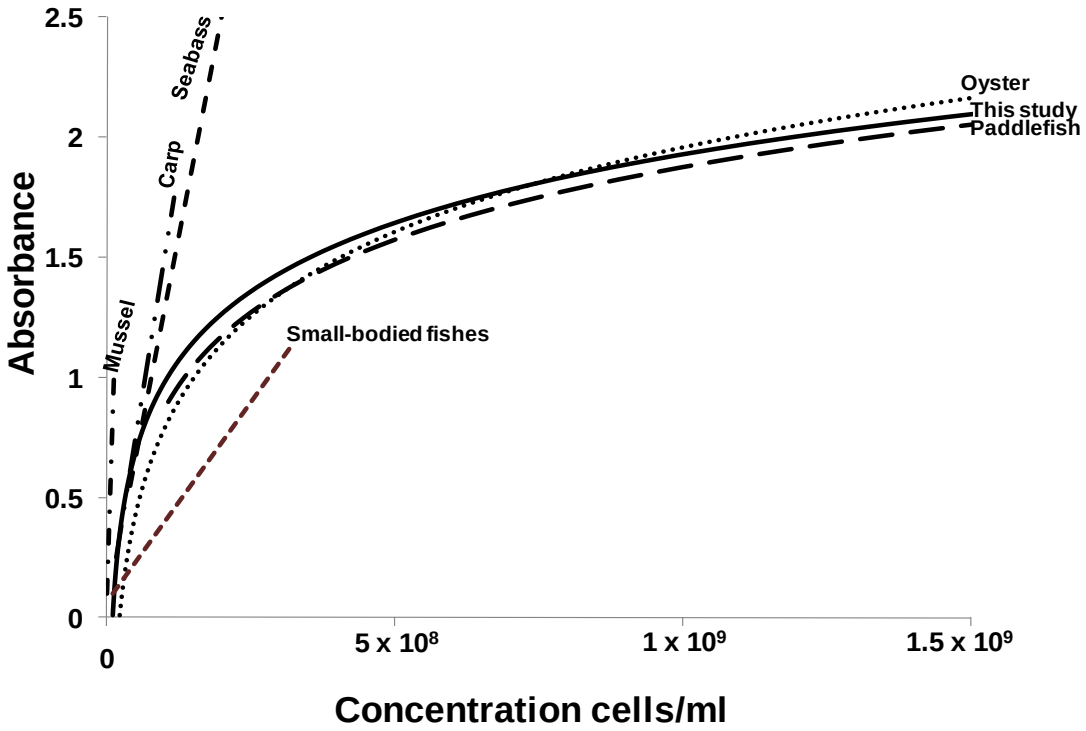

Figure 9. Relationships between absorbance and sperm concentration in previous studies: blue mussel (Del Rio-Portilla and Beaumont 2008), common carp (Takashima et al. 1984), seabass (Fauvel et al. 1999), Pacific oyster (Dong et al.), paddlefish (Linhart et al.), small-bodied fishes (zebrafish, medaka, and green swordtail) (Tan et al. 2010), and a multi-species curve (this study).

A spectrophotometer is generally composed of one or more light sources, a wavelength selector, sample container, detector, signal processor, and readout devices. In a turbidity measurement a spectrophotometer is used to measure the attenuation of light as a function of wavelength as it passes through a sample. Because previous studies used different wavelengths and different spectrophotometers, the instrument characteristics (such as light transmission properties of the sample cell, aperture size, distance between sample cell and detector, and pathlengths) were different. For example, cuvettes made of plastic or glass can be used to measure within the visible spectrum, while a cuvette made of quartz glass or fused silica should be used for the UV region (i.e., below 350 nm). In addition, there are several types of detectors such as photovoltaic cells, vacuum photodiodes, photomultiplier tubes, and silicon photodiodes (Cole and Levine 2003). Each detector has a differential sensitivity of wavelength range (Cole and Levine 2003). All of these components and configurations are instrument specific and constitute some of the known and unknown sources of variation in previous studies.

Previous studies in livestock compared the estimation of sperm concentration using different spectrophotometers. Comparison of duplicate instruments yielded nearly identical results in bull sperm (Foote 1972, Foote et al. 1978) while the use of different models of spectrophotometers resulted in different readings for the same sperm samples from boar (Knox et al. 2002, Knox 2004). It is important to note that one study compared three spectrophotometers for the sperm of boar, bull, and stallion (Rondeau and Rouleau 1981). Two of the instruments yielded no difference in standard curves, and the authors concluded that if spectrophotometers have the same characteristics in terms of spectral bandwidth, the calibration curves need not be statistically different. Even so, variable maintenance and calibration of instruments, different

types and quality of cuvettes, and the lifespan of instrument lamps could introduce differences in responsivity and accuracy across time for a single instrument or among duplicate instruments.

Feasibility of establishing a general curve:

Determination of an absorption spectrum for analyzing a given sample material is the first step of turbidimetry analysis to identify the maximum sensitivity for measurements. This is typically done by plotting the absorbance measurement as a function of wavelength (Dong et al. 2005a, Tan et al. 2010). Wavelengths in the range of 260-660 nm have been used to determine sperm concentrations in fishes. The livestock industry has used wavelengths in the range of 275 to 630 nm (Foote et al. 1978). Wavelengths in the range of 550 to 576 nm appear most sensitive for white suspensions (color induced absorbance) such as for sperm, and a wavelength of 550 nm is recommended and mostly used (Foote et al. 1978, Knox 2004). In the present study, we tested five wavelengths (400, 450, 500, 550 and 600 nm) with seven species. There was no single wavelength that yielded maximum absorbance. This indicates that any of these wavelengths could be appropriate to generate a standard curve.

Other studies found that lower wavelengths yield greater variation. For example, for zebrafish the maximum absorbance of sperm was around 265 nm but the absorbance profiles at wavelengths below 380 nm (UV) varied widely for single samples and the use of wavelengths above 400 nm was recommended (Tan et al. 2010). In a different study with blue mussels, the maximum absorbance was at 216 nm but the absorbance values had a large variation and the use of wavelengths above 320 nm was recommended (Del Rio-Portilla and Beaumont 2008). In contrast, another study recommended the use of 260 nm based on the hypothesis that differences in optical density using this wavelength in different fishes were due to the DNA content. This report compared the light absorption of sperm of turbot *Psetta maxima*, seabream *Sparus auratus*, and seabass *Dicentrarchus labrax* but without a standardized concentration (Fauvel et al. 2010). This hypothesis and other claims such as that volume changes in sperm can be tracked by their absorbance (Dzuba and Kopeika 2002), should be addressed in future research. In general, lower wavelengths yield higher transmittance values and thus have been recommended for use (e.g., Del Rio-Portilla and Beaumont 2008). Absorbance values below 0.1 and above 1.0 represent 10% and 20% transmittance, respectively, while absorbance values above 2.0 represent ≤1% transmittance. In earlier spectrophotometers, this low transmittance could result in inaccurate readings, although this is not normally a problem with current instruments (Mantle and Harris 2000). Earlier spectrophotometers used vacuum photodiodes while current spectrophotometers use silicon photodiodes and optical filters with higher resolution.

Overall, there are three main types of spectrophotometers: 1) Visible spectrophotometers that have inexpensive glass components, use tungsten lamps as the light source, and operate across a range of 325 to 1,000 nm. Older instruments of this type rely on blue- and red-sensitive phototubes. 2) Ultraviolet-Visible spectrophotometers that measure absorbance in the 200 to 1,000 nm range. For instruments of this type the most common source of radiation is the hydrogen-discharge lamp, but if more intensity is desired (3-5 times) a deuterium-discharge lamp is used. 3) Infrared spectrophotometers that use a heat source (i.e. Globar and Nernst glower) and the spectra result from molecular vibrational transitions in the range of 750–15,000 nm (Csuros 1997). These have been applied for multi-component analyses of fish meat (Elvingson and Sjaunja 1992) but have not been used for estimation of sperm concentrations.

In this study, we evaluated wavelengths within the visible spectrum (390–750 nm). This is the first study that attempted to evaluate a general standard curve to measure sperm

concentration for fish species. Although there is a publication in which a single formula was used to measure sperm concentration in the Siberian sturgeon *Acipenser baerii* and sterlet *A. ruthenus*, there was no explanation of the reasoning behind this usage, or if both species were validated for that equation (Sarosiek et al. 2004). It has been stated as a common belief that different calibration curves are required for different species because of the specificities of the sperm (Foote et al. 1978). However, as stated above, a general standard curve has been established for bulls, boars, and stallions (Rondeau and Rouleau 1981), and there were no differences among the slopes of the calibration curves for these livestock species. Whether for mammals or for fish, seminal samples consist of seminal plasma (or seminal fluid) and spermatozoa. Fish seminal plasma contains mainly mineral compounds and low concentrations of organic substances (Ciereszko et al. 2000a). The absorbance of seminal plasma (< 0.1) was measured in seabass and yellowtail flounder *Limanda ferruginea*, which led to the conclusion that seminal plasma did not interfere within the wavelength range (200–900 nm) tested (Clearwater 1996, Fauvel et al. 1999). In fact, the effect of light scattering and light absorption by spermatozoa has been shown to dominate the effect of light absorption by seminal plasma (Rothschild 1950, Taneja and Gowe 1961). Therefore, in the present study there was little justification to remove the seminal plasma before estimating the concentration by the use of spectrophotometer. We hypothesized that a single calibration curve could be used to determine the concentration of spermatozoa for most fish species.

In this study, after comparing the data of all fishes, the correlation coefficient of the standard curve at all wavelengths was higher than 0.77. But the validations of the observed values against the spectrophotometric estimation were significantly different. This was resolved however when the collection methods (stripped and crushed) were separated. The correlation coefficients for all species without catfish were higher ($R^2 = 0.87$) and there was no statistical difference between observed and estimated values at wavelengths higher than 450 nm. Differences in absorbance between sperm collection methods (stripped and crushed) have been reported in zebrafish and green swordtail *Xiphophorus helleri* (Tan et al. 2010), although a robust applicability was reported in that study among all of the curves generated across species (zebrafish, swordtail, and medaka *Oryzias latipes*), and different collection techniques (stripped and crushed). For relatively pure, homogenous sperm samples, if the sperm size and shape among species are similar, the changes in light absorbed among samples will primarily be due to a difference in sperm cell concentration (Rondeau and Rouleau 1981). In addition, studies have shown that dilute suspensions of most bacteria have nearly the same absorbance per unit of dry weight concentration, regardless of the variation of cell size and shape (Omstead 1990). Therefore, based on our results, we postulate that a general standard curve for any single instrument should be able to measure concentration for most fishes from which pure samples of sperm can be collected.

To evaluate the effect of other cell types such as those in whole blood, sperm with no overt blood contamination was collected in catfishes. When these absorbance values were combined with the data for all species without blood, the resulting correlation value was the same as that observed for all fishes collected by stripping. When there was no addition of blood to the samples, the absorbance was directly related to the sperm concentration. Therefore blood cells and other cell types that are mixed with sperm during crushing of the testis can interfere with accurate estimation of sperm concentration. Depending on the timing in relation to the spawning season, the ratio of somatic cells and germ cells can vary considerably. Crushing of the testes can release a mixture of cells types such as spermatogonia, spermatocytes, spermatids,

spermatozoa, and Sertoli-like cells (Viveiros 2003). Failure to properly clean the testes before crushing could also contaminate samples with connective tissues that contain Leydig-like cells, nerve fibers, fibroblasts, collagen fibers, smooth muscle cells, and endothelial cells (Grier and Uribe 2009). In addition, the cytoplasm of epithelial cells of the main testicular ducts and spermatic ducts contains lipid vacuoles, and the seminal fluids also contain lipids during interspawning periods (Lahnsteiner and Patzner 2009). Based on our observations contamination of this sort can lead to a systematic overestimation of sperm concentration in direct relationship to the volume of blood or other contaminants present as described above (Figure 7). Other studies found similar effect, when debris present in the raw semen, such as cytoplasmic droplets, affected the accuracy of the spectrophotometric method (Christensen et al. 2004). Thus, depending on spawning condition the correlation between absorbance and sperm concentration could be affected by somatic contamination, and more work needs to be done to evaluate measurements of samples collected from crushed testes at different times of the year. It should be noted that microscopic observations can be used to assess the level of contamination of samples before measuring the absorbance (Figure 3).

This is not the first publication to mention that other cell types such as blood can disturb absorbance measurements. In fact, the presence of other cells in the sperm of the landlocked sea lamprey *Petromyzon marinus* disrupted the use of spectrophotometry (Ciereszko et al. 2000b). Stripped samples of the African catfish *Clarias gariepinus* were contaminated with blood and the turbidity estimation for sperm concentration could not be applied (Viveiros et al. 2003). And, in African catfish two types of sperm samples, "white" and "grey", were collected by dissection and stripping of the testes. The white samples had a higher sperm cell concentration and absorbance values (650 nm) than did grey samples (Mansour et al. 2004). The white samples were characterized by high sperm densities and a low number of spermatids, while the grey samples contained numerous germinal cysts with spermatids in addition to sperm. In another study, different levels of blood contamination of rainbow trout *Oncorhynchus mykiss* milt were obtained in relation to sampling period and method of milt collection (Ciereszko et al. 2004). In an attempt to measure the influence of blood in sperm samples, blood was added to a pure sample (final volumetric proportion of blood was 0.2%) of rainbow trout sperm (sperm concentration 9×10^9 to 1.4×10^{10} cells/mL) (Ciereszko et al. 2004). Sperm quality parameters (osmolality, protein concentration, lactate dehydrogenase activity) were not affected by the contribution from this small amount of blood and although this study used turbidity methods it did not report any influence of blood on the estimation of sperm concentration (we presume due to the high proportion of sperm cells in relation to the erythrocytes).

In the present study, five different volumetric proportions of blood were tested with three concentrations of sperm. Although the absorbance values for the different volumes of blood were different, there was no statistical difference in the standard curves for wavelengths higher than 450 nm. This corresponded with the sperm collected by crushed testes in which higher correlations were obtained at wavelengths from 500 to 600 nm. Contamination with as much as 1% blood, did not affect the standard curve for concentrations as low as 1×10^6 cells/mL. This indicates that blood contamination might not be a major factor of concern when measuring concentration by spectrophotometer in samples with a high proportion of sperm cells in relation to blood cells (Figure 7, lower panel). It could however be expected that higher proportions of blood to sperm (> 1%) at low sperm concentrations would influence the absorbance, especially at the wavelengths of maximum absorbance of blood (> 450 nm) which corresponds to the peaks of maximum absorbance of oxyhemoglobin (540 and 575 nm) (Stryer 1995).

The effects caused by crushing of testes with respect to contamination of the samples are not simple or straightforward. Errors in absorbance or sperm concentration can result in overestimations or underestimations. For example, use of a calibration curve developed during the peak of the spawning season using pure sperm when applied to crushed testis samples could yield overestimations of concentration (based on the inflated absorbance values of the samples). Or, conversely use of a curve developed early in the spawning season when testes did not contain large volumes of mature sperm would result in an upward-shifted curve (based on higher somatic absorbances) and could yield underestimates in concentration later in the spawning season when sperm production peaks. Thus, the types of errors are affected by variations of cell types in relation (proportion) to one another and this relationship varies over time due to reproductive seasonality. As such, the observed effects can sometimes be small between crushed testis and pure sperm samples (e.g., Figure 8), but the potential contribution of cells such as erythrocytes to absorbance should not be overlooked. It is also important to note that the patterns observed in the absorbance profiles generated by blood addition (Figure 7) do not exactly match the absorbance profile of pure blood (Figure 8) likely due to the relative contributions of the various components in contaminated samples (as described above) to the aggregate profile. Hemoglobin itself can exist in a variety of forms with different absorbance profiles based on interactions with atmospheric gases (Stryer 1995).

The testes of ictalurid catfishes in particular present an even more complicated picture with regard to contamination caused by crushing. The testes possess two recognizable portions: anterior and posterior (Figure 10) (Sneed and Clemens 1963). These two portions are characterized by different cells types, and their relative sizes and color vary depending on the spawning period. The anterior portion is considered to be spermatogenic in function and is small, flat and transparent outside of the spawning season, but expands dramatically, turning white with finger-like extensions, and composing 2/3 of the testis, as sperm production fills the tubules and lumen (Guest et al. 1976). This portion contains the majority of available sperm and is sometimes the only portion of the testis to be harvested while the posterior portion is discarded (Tiersch et al. 1994). The posterior portion has been reported to function as an accessory glandular organ that secretes a mucopolysaccharide-protein-lipid-rich fluid (seminal vesicular fluid) believed to contribute to the seminal volume and participate in prolongation and stabilization of sperm viability (Chowdhury and Joy 2007). The posterior testis is composed of interstitial cells, fibroblasts, blood capillaries, and nerve elements. During spawning the epithelial cells of the posterior portion contain an abundance of rough endoplasmic reticulum, Golgi apparatus, secretory vacuoles, and electron-dense secretion products (Chowdhury and Joy 2007). The role of the posterior portion is most likely involved in maturation and nutrition of sperm, although it possesses a similar sperm concentration (based on cells per wet weight of tissue) as the anterior (Guest et al. 1976, Jaspers et al. 1978). The size and color of the posterior portion is more variable than the anterior portion (our unpublished observation) and it can be larger or smaller than the anterior portion, although it is almost darker (pink to light red to brown).

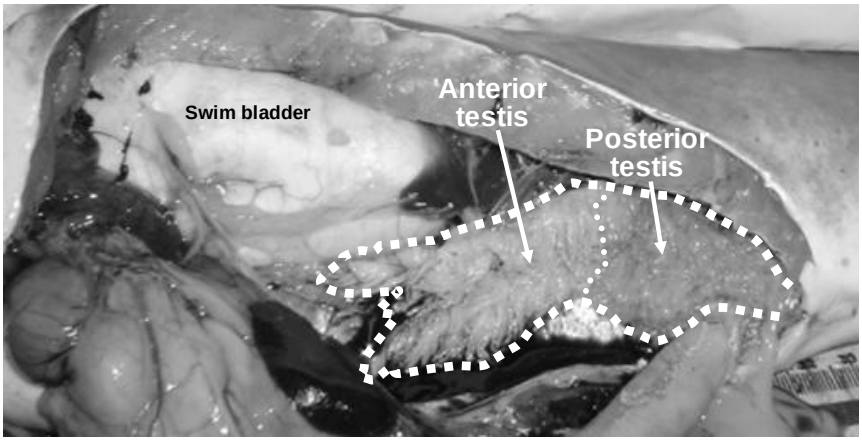

Figure 10. View of channel catfish testes within the body cavity during the spawning period (April to May in Southern Louisiana). The anterior portion is considered to be spermatogenic and the posterior portion to serve glandular functions. Dissection ahynd crushing of the testis to collect sperm can involve both portions or only the anterior portion, and can contribute a variety of somatic cells and other contaminants to samples

As indicated above, sperm collection can proceed by crushing of the anterior portion alone, or as an admixture with the posterior portion yielding a complex collection of cell types and compounds within the sample with potential for considerable effects on the aggregate absorbance values. In this study, we chose to crush and mix both portions of the testes to fully capture the variation encountered in different protocols. From the previous discussion it should be evident that development of calibration curves from either portion or their combination would present considerable variation throughout reproductive seasonality and offers great opportunity for standardization of protocols and reporting to reduce or minimize variation within and among studies and hatchery operations. Future studies are needed to more fully evaluate these portions of the ictalurid testis in terms of biological function and the methods appropriate for their use in practical spawning protocols (including cryopreservation).

Protocols describing methodology to use turbidity to measure sperm concentrations have been published previously for livestock (Foote 1972, Foote et al. 1978), and a recent study was performed to illustrate development and standardization of photometric measurement of sperm concentration in Pacific oysters *Crassostrea gigas* (Dong et al. 2005a). Procedurally, care should be taken when collecting sperm, because contamination with other substances such as urine could affect calibration and concentration estimates (Clearwater 1996). The viscosity of the sperm often makes it difficult to obtain a homogeneous dilution of the sample, and depending on the pipette and tips used for sample handing, different values could be obtained from the same sample. Therefore thorough mixing of sperm suspensions before measurement is essential for accurate readings (Cabrita et al. 2009). The presence or absence of small aggregations of spermatozoa in an aliquot of dilution could affect the accuracy and precision of sperm concentration estimates (Rakitin et al. 1999). These types of errors are magnified when working with the limited sperm samples (2-4 µl) collected from small-bodied fish such as zebrafish (Tan et al. 2010). Standard curves should be established for each spectrophotometer and regular calibrations are needed to ensure accurate estimation of sperm concentration (Knox et al. 2002). In the present study, standard curves generated at wavelengths from 450 to 550 nm, within the range of 1×10^6 to 6×10^{10} cells/mL were effective for determination of the concentration of sperm from paddlefish, red drum, tilapias, white bass, and striped bass, and would likely be

useful for other fish species. Data for sperm concentration should be logarithmically transformed prior to application of simple linear regression. Such log transformation has been suggested previously (Berman et al. 1996, Handelsman 2002) and applied in Pacific oysters (Dong et al. 2005a), yellowtail flounder (Clearwater 1996), paddlefish (Linhart et al. 2000), and blue catfish (Campbell et al. 2005a). It is important to note that the generation of a standard curve will be specific for each type of spectrophotometer or instrument and even different laboratories, because not all conditions can be expected to be the same among locations (Knox et al. 2002). Accurate and comprehensive reporting of methods is thus necessary to accurately evaluate and compare studies.

Although the hemacytometer is considered as the "gold standard" for measurement of sperm concentration, there are variations among different designs and operators (Seaman et al. 1996, Christensen et al. 2005). Estimation of sperm concentration from a spectrophotometric determination of turbidity is routinely used in artificial insemination of mammals because it is fast and precise (Prathalingam et al. 2006). The precision of turbidity estimation derives from standard curves produced from multiple hemacytometer counts. Although some have considered spectrophotometer to be costly and time consuming (Powell 2002), there is little evidence to support this observation. Compared to a hemacytometer there is a higher initial cost for the spectrophotometer (Table 6). However, the same spectrophotometer can be used for other purposes such as measuring water quality or general laboratory analyses. There are portable spectrophotometers that are used in the daily routine of fish farming, and could provide an inexpensive and rapid method for sperm concentration determination in field conditions (Dietrich et al. 2005a, Dong et al. 2005a). The use of a portable spectrophotometer (Eppendorf, Germany) has been reported to estimate the sperm concentration in whitefish (Ciereszko et al. 2008). Other options are microspectrophotometers that work with microliter sample volumes. These microspectrophotometers can be essential to measure sperm concentration for small-bodied fishes such as zebrafish that only yield 2-4 µL total of sperm sample (Tan et al. 2010).

Other techniques available to estimate sperm concentration include computer-assisted sperm analysis (CASA) instruments (e.g., Hamilton Thorne, Beverly, MA, USA; SQA-V Medical Electronic Systems, Los Angeles, CA, USA), flow cytometry, and fluorescence microscopy, and Coulter counter. These techniques require specialized instruments that are prohibitively expensive if they are used only to measure sperm concentration. A disadvantage for using CASA is that it often requires a specific disposable chamber in which non-uniform distribution of the spermatozoa can lead to false estimations (Lu et al. 2007). There is no replacement for a direct observation of the sperm to detect other sperm quality parameters such as morphology and the presence of clumps or other types of contaminants. There is image analysis software available for free downloading distributed by the National Institutes of Health (*rsbweb.nih.gov/ij*) that has been used to estimate sperm concentration in Atlantic bluefin tuna *Thunnus thynnus thynnus* (Mylonas et al. 2007).

Table 6. Comparison of price, volume requirements, and wavelengths available for different spectrophotometers.

Spectrophotometer	Cost (US$)	Volume needed	Wavelength range (nm)	Web address
Ultrospec 10, GE HealthCare[1]	742	1.5 ml	600	www.gelifesciences.com
Biowave, Biochrom WPA[1]	762	10 µl	190 - 900	www.biochrom.co.uk
YSI 9300[1]	807	10 ml	450 - 650	www.ysi.com
Smart2, LaMotte[1]	909	10 ml	350 - 1000	www.lamotte.com
DR890 Colorimeter, Hach[1]	1,177	2.5 ml	420 - 610	www.hach.com
Genesys 20, Thermo Scientific[1]	1,862	1 ml	325 - 1100	www.thermo.com
590b Densimeter, ARS[2]	1,895	200 µl	ND[3]	www.arssales.com
BioPhotometer, Eppendorf[1]	4,585	50 µl	230 - 650	www.eppendorf.com
GeneQuant, BioChrom[1]	5,071	7 µl	190 - 900	www.gelifesciences.com
Epoch, BioTek[1]	8,950	2 µl	200 - 999	www.biotek.com
NanoDrop, Thermo Scientific[1]	8,950	0.5 µl	190 - 840	www.nanodrop.com

The use of flow cytometry to measure sperm concentration has yielded variable results (Lu et al. 2007, Anzar et al. 2009) and depends on how the counts are performed (i.e., typically in relation to a known concentration of a fluorescent bead internal standard), the concentration range tested, and whether the emphasis is on precision or accuracy (Haugen 2007). Future research needs to be conducted to compare newer designs of flow cytometers (such as the Accuri C6®, Ann Arbor, MI, USA) which measure the actual volume that is pulled from the sample and can directly estimate cell counts without the need of fluorescent bead standards. Another new flow cytometry device (S-FCM, Kobe, Japan) was developed to measure sperm concentration. This device has been reported to be suitable for measurement of human sperm concentration (Tsuji et al. 2002). A recent publication demonstrated that computer-aided fluorescent microscopy (NucleoCounter SP-100, Denmark) could be used to measure sperm concentration in brook trout *Salvelinus fontinalis* (Nynca and Ciereszko 2009). Coulter counters have long been accepted as a reliable technique for particle sizing and counting (Brillard and McDaniel 1985) and are available in some fish hatcheries for ploidy determination (Wattendorf 1986), but are expensive for sperm counting only. Other techniques such as cell-UV chambers, and packed cell volumes (e.g., VoluPAC, Sartorius, Germany) need to be evaluated for aquatic species. Potentially, a general standard curve for fishes could be incorporated into analysis-specific spectrophotometers (e.g., 590B Densimeter, Animal Reproduction System, Chino, CA, USA). Such densimeters are designed to measure sperm concentrations of stallions, bulls, boars, canines, roosters, and turkeys.

Conclusions

We conclude that estimation of sperm concentration is essential for many studies in aquatic species and that reports using sperm estimations from spectrophotometric determination of turbidity should include at a minimum: the spectrophotometer model and type, cuvette description, wavelengths used, absolute determination method (e.g., hemacytometer) used, range

of sperm concentrations tested, absorbance range tested, the standard curve, linear regression equation, and the coefficient of determination. In this study, a single general (instrument-specific) standard curve generated at wavelengths of 450 to 600 nm within the range of 1×10^6 to 6×10^{10} cells/mL was developed for determining the concentration of sperm from seven different fish species where sperm was collected by stripping. With further study or procedural modifications this could apply to fishes for which the testes are crushed. This would have broad applicability in reproductive studies and is essential for standardization of cryopreservation.

The importance of sperm concentration determination in livestock has been recognized since 1939 (Comstock and Green 1939). Sperm concentration is vital in artificial insemination because the number of sperm determines how many females can be inseminated (Foote 1972). The livestock industry sells packaged semen for artificial insemination of livestock in "frozen doses" that supply a specific number of sperm. This requires a high level of strict quality control and standardization for all of the parameters involved in the dose, including sperm concentration. Standardization and reproducibility are key factors for the success of this industry for livestock (Thibier and Wagner 2002).

Sperm cryopreservation is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding. In addition, the availability of frozen sperm allows the creation of genetic resource repositories and conservation programs to increase the potential breeding population size to ensure that proper genetic combinations are produced in breeding of endangered species (Tiersch 2008). The future development and utility of technologies such as cryopreservation will rely on standardization and control of major variables such as sperm concentration. The results presented in this chapter call attention to the need for standardization and suggest that variation in sperm concentration results can be influenced more by the instrument used than the species studied.

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New Chapter

Flow Cytometry for the Assessment of Sperm Quality in Aquatic Species

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Introduction

The use of fluorescent staining in conjunction with flow cytometry for the assessment of sperm quality in aquatic species has increased over the past decade. However, in contrast to the common use of flow cytometric assessment of sperm quality in mammalian species (i.e., hundreds of published studies), the use of this technique in aquatic species is relatively unexploited but becoming increasingly important. The increase in usage has been largely driven by the adaptation and adoption of sperm quality assays developed for use in mammalian species, which are vast in number compared to the range currently applied in aquatic species (Martinez-Pastor et al. 2010). Most assays developed in mammalian species are designed to target specific sperm structures or processes that are often sufficiently conserved across species to allow application in aquatic species. In addition to the assays adapted from mammalian species, more specialized sperm quality assays have been optimized in aquatic species for biomonitoring applications (e.g., Jenkins et al. 2010) and these have great potential for use in sperm quality assessments for cryopreservation.

Among the sperm quality assays that are currently used in aquatic species, assays for sperm membrane integrity and mitochondrial membrane potential have had the greatest application. One of the most commonly used in aquatic species is the SYBR 14/ propidium iodide (PI) assay for plasma membrane integrity, often referred to as a "sperm viability" assay. Several studies have used this assay to assess sperm quality in fresh and post-thaw samples to evaluate the success of cryopreservation protocols (e.g., Cabrita et al. 2005, Paniagua-Chávez et al. 2006). This assay has also been used in environmental toxicity studies to determine the effect of exposing sperm to antibiotics (Segovia et al. 2000) and herbicides (Favret and Lynn 2010) in a range of vertebrate and invertebrate aquatic species. Another commonly tested parameter in sperm from aquatic species is mitochondrial function, which is assessed using mitochondrion-specific fluorescent dyes that accumulate in proportion to membrane potential. The most commonly used mitochondrial dye in aquatic species is rhodamine 123, which is often combined with PI to enable concurrent assessment of membrane integrity (e.g., Ogier de Baulny et al. 1997, Liu et al. 2007), but a few studies have used other mitochondrial stains such as MitoTracker Red (Favret and Lynn 2010) and JC-1 (Guthrie et al. 2008).

In addition to the often kit-based assays adapted from mammalian species, assays that are currently used in aquatic species for biomonitoring applications also have potential utility in sperm quality assessments for cryopreservation, including assessment of DNA integrity (Jenkins et al. 2010) and ploidy analysis (Psenicka et al. 2009). These assays use fluorescent dyes that label DNA, such as PI or 4',6-diamidino-2-phenylindole (DAPI), and are typically used to assess the effect of environmental toxins on spermatogenesis and subsequent sperm quality. Although they require a greater level of flow cytometric expertise than the kit-based assays and require research for validation studies, they offer additional means of assessing and predicting the effect of cryopreservation on sperm survival and fertilizing ability.

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Considerations for Flow Cytometry in Aquatic Species

As the use of flow cytometry for sperm quality assessment in aquatic species continues to increase, it is important to ensure that a consistent approach is taken to the application of new and existing assays. The main factors that should be considered and reported when analyzing sperm quality by flow cytometry are outlined in Table 1, and discussed briefly below.

Table 1. Suggested minimum reporting requirements for sample preparation and flow cytometric analysis of sperm from aquatic species.

Parameter	Information required
Sample storage	Temperature and duration of sample storage after collection
Sperm concentration	The number of cells per unit volume (e.g., sperm cells/mL)
Staining conditions	Final concentrations of each dye to which sperm were exposed, expressed in molar units (e.g., nM, µM)
	Staining volume (i.e., the volume of sample to which stains were added)
	Staining temperature (e.g., room temperature, on ice, 4 ºC)
Staining time	Total staining time for each dye used
	Time between staining and analysis (i.e., how long after the staining time samples were analyzed)
Collection parameters	Number of events (or volume) collected
	Basis for event counts (i.e., based on FSC/SSC, gated, or fluorescent counts)
	Flow rate
Gating parameters	Identification of the sperm population
	Method for exclusion of debris from analyses
Reporting of results	Concentrations where possible (e.g., concentration of intact cells)
	Basis for calculation of percentage data (e.g., percentage of the total or gated, event count)

Although only a few flow cytometric assays are used regularly for sperm quality assessment in aquatic species, direct comparison of results among studies is problematic, if not impossible, due to the large amount of variability in sample preparation, staining, and flow cytometry protocols. For example, among 15 studies that reported membrane integrity data using the SYBR 14/ PI assay in aquatic species in the past 10 yr, there was considerable variation in, or failure to report, sperm concentration, dye concentration, collection parameters (e.g., the number of events collected, flow rate, and population gating), and temporal components such as the time between collection or thawing and assessment, duration of treatments, and time between staining and flow cytometric assessment. This variability is in part due to limited access to flow cytometers for aquatic species research, with researchers often having to analyze samples at facilities focused on the analysis of humans or livestock. This has meant that researchers working with aquatic species often have limited opportunity to gain significant flow cytometry experience, and therefore must rely on the expertise of mammalian researchers who usually have minimal experience working with non-mammalian samples. While the past problems and variations in the use of this technology are understandable, the same approach cannot be used going forward. A standardized approach to sperm quality assessment for aquatic species is urgently required for research and eventual industrial application (Leibo 2000). There are many factors that can affect the quality and effectiveness of flow cytometric analyses, and all must be taken into account to allow accuracy and reproducibility of results.

Perhaps the most important factor in sperm quality assessment is sperm concentration, which aside from being a measure of quality in itself, is also relevant to all other measures of quality. The measurement and control of sperm concentration is extremely important for artificial fertilization and cryopreservation in aquatic species (Dong et al. 2007), but is usually not recognized, nor is it considered when assessing sperm quality. As such, flow cytometry results are typically reported as percentage data with no indication of the actual number of competent cells within a sample. It is important to note that percentage data are only useful in the context of concentration, i.e., proportions must be considered relative to the whole (put simply, 50% of 10^8 cells is much different from 50% of 10^6 cells). This is particularly important to consider when assessing thawed sperm samples. Cryopreservation and related processes (e.g., cryoprotectant exposure) can damage or destroy sperm, affecting concentration but not necessarily the proportion of intact cells, and this would not be detected with percentage data alone. Although traditional flow cytometry systems cannot calculate sperm concentration directly, concentration can be calculated by the addition of a known number of fluorescent counting beads to a sample, or separately by hemocytometer counts or spectrophotometry. Newer flow cytometry systems, such as the Accuri C6 (*www.accuricytometers.com*), are able to measure the sample volume collected, enabling direct calculation of concentration, and it is likely that this feature will be increasingly incorporated into future flow cytometry.

When percentage data are reported, it is important to define how the percentages are calculated as this can greatly affect the outcome and accuracy of results. For example, the proportion of intact cells is likely to be much lower when expressed as a percentage of the total event count (i.e., all forward scatter (FSC) vs. side scatter (SSC) events, including debris and other non-sperm events) than as a percentage of the gated event count (i.e., the events identified as the sperm population). This is particularly important for species that require dissection and crushing of the testes for sperm collection, as is the case for several commercially important aquatic species, including channel catfish *Ictalurus punctatus* (Christensen and Tiersch 2005) and Pacific oysters *Crassostrea gigas* (Dong et al. 2005), and small-bodied biomedical model species such as zebrafish *Danio rerio* and swordtails (*Xiphophorus* species) (Yang and Tiersch 2009). Sperm samples collected by dissection and crushing of the testes typically contain significant contamination with blood and other somatic cells, which will affect total event counts and present difficulties in gating and subsequent percentage calculations. It is therefore important to provide details on how the sperm population was identified from within the overall accumulation of events, and report gating parameters that were used to exclude non-sperm events from analysis (e.g., Figure 1). This information is essential to ensure accurate interpretation and reproducibility of the reported procedures.

In addition to sperm sample considerations, consistent control and reporting of staining and flow cytometry settings is required to enable comparison among studies. At present, there is a great deal of variability in staining and flow cytometry methodology among studies. For example, of the 15 studies in the past decade that reported flow cytometry data using the SYBR 14/ PI assay, 11 reported various combinations of 5 concentrations of SYBR 14 (10 – 400 nM) and 3 concentrations of PI (10 – 14 µM), while the other 4 studies only reported dilution ratios or volumes of stain added to the samples. When this is combined with the inconsistencies in the amount of sperm to which the dyes were added (variously reported as concentration ranging from 1×10^6–1×10^9 sperm/mL, or dilution ratio ranging from 1:6-1:99) this leads to immense variation in the sperm:dye ratios among these studies. Although some amount of variability in methodology among different species or laboratories is to be expected, such large differences

make direct comparison of results or methodologies among studies virtually impossible, especially when dilution ratios or volumes are used with no indication of starting or final sperm or dye concentration.

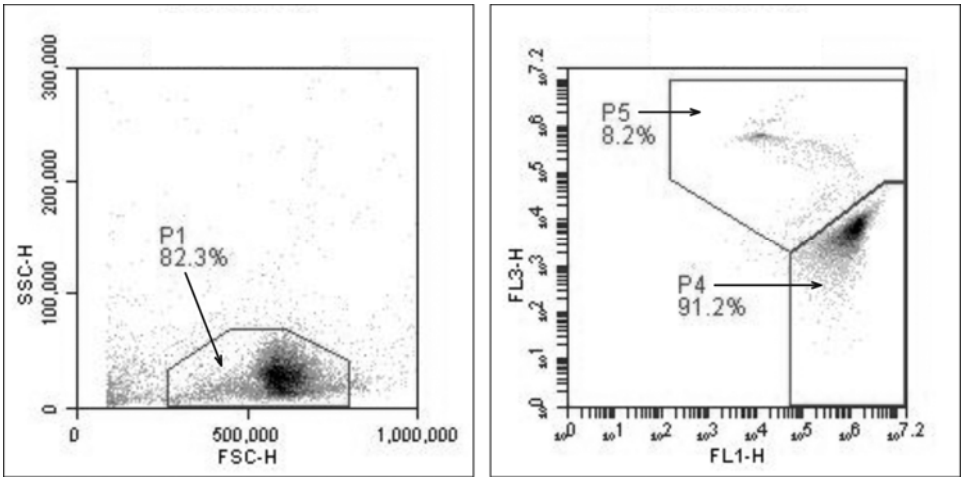

Figure 1. Forward scatter vs. side scatter (FSC vs. SSC, left panel) and FL1 vs. FL3 (SYBR 14 vs. PI, right panel) scatter plots of zebrafish sperm in 300 mOsmol/Kg HBSS. The region designated as "P1" in the FSC vs. SSC plot is the gated sperm population, and events falling outside this region are debris. The region designated as "P4" in the SYBR 14 vs. PI plot is the intact sperm population (i.e., sperm with intact plasma membranes), and those falling in the "P5" region have damaged plasma membranes.

One of the main potential sources of error in flow cytometric analysis of sperm samples arises from how the samples are analyzed. Although reports often state how many events (discrete electronic signals corresponding to target cells or other materials depending on instrument settings) were analyzed from a particular sample (typically 10,000 or more), information on how these events were counted or the flow rate at which the events were collected is often omitted. For example, 10,000 events based on FSC vs. SSC will be different to 10,000 events based on the gated sperm population, and this difference will be magnified in samples that were collected by dissection and crushing of the testes. In addition, the accuracy of these counts will be affected by the flow rate. If the flow rate is too fast or the sample is too concentrated, the sensitivity and precision of the flow cytometer are affected (Shapiro 2003) and the ability of the software to distinguish between individual events and between target cells and debris is greatly reduced. Consequently, the ability to distinguish and gate around target cells to exclude debris in the resulting scatter plots is reduced, affecting the accuracy of data analysis. This once again emphasizes the importance of measuring and controlling sperm concentration when analyzing sperm quality by flow cytometry, and highlights the need to consider and report data collection settings when reporting flow cytometric methodology.

Another major factor to consider when analyzing sperm quality by flow cytometry is temporal variability, which is relevant to all activities ranging from sperm collection to quality assessment. For flow cytometry, it is important to report how long after collection or thawing the samples were analyzed, and the total duration of exposure to fluorescent stains prior to analysis. Although minor variations in the time between collection and quality assessment may not significantly affect the results obtained for fresh samples, temporal effects are likely to have a

greater impact on aged, thawed, or poor quality samples. Sperm that have been thawed after cryopreservation are generally of lower quality than fresh, unfrozen sperm, and therefore may degrade faster than fresh sperm and make accurate comparisons between treatments or individuals difficult.

While the factors mentioned should be considered for all sperm samples, they are particularly important when analyzing thawed samples. As mentioned above, cryopreservation and related processes can weaken or destroy sperm. This can affect the flow cytometric data by decreasing the concentration of sperm in the sample, and by increasing the amount of debris present. This can in turn affect the accuracy of collection parameters (if collecting a set number of events) and the calculation of percentage values. In addition, thawed sperm may be more susceptible to temporal effects, such as variations in the time between thawing and analysis, which could further affect the results. It is for these reasons that cryopreserved sperm samples cannot be considered to be the same as fresh sperm samples, and care must be taken to account for these factors when collecting and comparing flow cytometric data from fresh and thawed sperm for assessment of cryopreservation effects.

Future Applications of Flow Cytometry in Aquatic Species

As sperm cryopreservation in aquatic species moves towards high-throughput applications for aquaculture and repository storage of genetic material (NIH-NCRR 2007), standardization of sperm quality assays and minimum reporting standards will become even more important. For aquaculture, it will be important to provide accurate assessments of sperm quality of the males selected for genetic storage and to allow end users (e.g., hatchery producers or farmers) to have confidence in the final cryopreserved product and enable efficient usage of genetic material. Information on the quality of samples from endangered species stored in genetic repositories will be important to ensure that high quality samples are preserved for future use, and for accurate calculation of sperm-to-egg ratios to ensure that valuable gametes are not wasted by using poor quality sperm for fertilization or by using more sperm than are required for a particular batch of eggs.

A standardized approach will be required for future assessments of sperm quality using existing assays, such as membrane integrity and mitochondrial membrane potential, and for the development of new assays specifically for aquatic species. Over the past few years there has been an increase in biochemical research, such as proteomic analysis, in sperm from aquatic species (e.g., Li et al. 2010) and it is possible that work such as this will lead to development of new assays for sperm quality. It is essential that minimum standards for reporting of flow cytometry data be applied to all studies on sperm quality. By controlling and reporting these factors, researchers can ensure the accuracy and reproducibility of their data, allow others to replicate or build on that work, and ensure that flow cytometry can be utilized and interpreted correctly for gamete quality in aquatic species as it is currently used in mammalian species.

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Measurement of Milt Quality and Factors Affecting Viability of Fish Spermatotzoa

Dale C. Honeyfield and William F. Krise

Overview

A series of experiments were conducted with Atlantic salmon *Salmo salar*, steelhead trout *Oncorhynchus mykiss* and lake trout *Salvelinus namaycush* to investigate the utility of flow cytometry to measure sperm quality. We also examined the effect of storage and cryopreservation media type, osmolality and urine contamination on sperm cell viability. The use of carboxyfluorescene diacetate for mitochondrial activity and propidium iodide to indicate outer membrane integrity were good indicators (r = 0.95) of fertilization ability. Osmolality was variable in milt and seminal plasma from the three species of fish examined. Osmolality of cryopreservation media and post-thaw media affected percent live sperm in cryopreserved samples of steelhead trout. Milt from Atlantic salmon males with non-motile sperm had lower measurable seminal plasma osmolality, sodium and chloride than did milt from males with motile sperm. Urine contamination decreased the percentage of live sperm. From these studies, osmolality of milt, seminal plasma and diluents were found to be important in maintaining high sperm quality.

Introduction

Viable sperm is an essential component in any successful animal production operation. Sperm quality in fishes is typically monitored by determining the number of motile sperm, the success in fertilizing eggs, or by measuring some aspect of cell metabolism. Without viable sperm, egg fertilization and embryo production will not occur. Cryopreservation does not improve sperm quality (Lahnsteiner et al. 1992, 1996); sperm motility and fertilization rate will be unchanged or decreased after the cryopreservation process. Thus it is essential to have a simple, rapid method of determining quality of stored sperm in order to maximize reproduction.

Poor sperm quality may result from the effects of genetics, diet, environmental stress (toxicants, water quality, fish density) or disease. The most reliable indicator of sperm quality is egg fertilization. However, the length of time required to obtain fertilization data for a sperm sample precludes use of this method as a quick screening tool. There is also a confounding range of results due to problems associated with the eggs. Therefore, some physical measurement on either the whole sperm cell or selected components of sperm is generally used to predict fertilization rate. Sperm motility is frequently used (Stoss and Holtz 1983, Stoss and Refstie 1983, Piironen 1993, Tiersch et al. 1994, Lahnsteiner et al. 1995, Koupal et al. 1995), but other methods are available.

Flow cytometry has been successfully used to determine sperm quality in cattle (Graham et al. 1990) and rainbow trout *Oncorhynchus mykiss* (Ogier de Baulny et al. 1997). However, Schneider (1993) measured mitochondrial function using carboxyfluorescene diacetate with flow cytometry method, but was unable to predict sperm quality in rainbow trout and white sturgeon *Acipenser transmontanus*. Research by Graham et al. (1990) and Ogier de Baulny et al. (1997) combined sperm with two fluorescent dyes, propidium iodide and rhodamine 123. If the sperm membrane is intact, propidium iodide is excluded from the cell and will not come in contact with

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cellular DNA. Compromised cell membranes do not exclude propidium iodide and a red fluorescence is detected. By adding a sec dye, rhodamine 123, the functionality of the mitochondria can be determined. Functional mitochondria will fluoresce green. Generally, the flow cytometer is set to count 10,000 cells and classify them as the percentage viable cells (green color), non-viable cells (red color) or transitional (cells with both red and green colors). The use of the two dyes provides significant information on the condition of the sperm cells.

Measurement of a component within the sperm or seminal plasma also has been used to predict sperm quality (Baynes et al. 1981, Piironen 1985, Lahnsteiner et al. 1996, Lin and Dabrowski 1996). Lahnsteiner et al. (1996) listed an array of seminal plasma and sperm cell constituents (potential metabolic substrates, metabolites, enzymes) used in their studies with rainbow trout. These authors concluded that seminal plasma osmolality, pH, and triglycerides were predictive of sperm quality. Seminal plasma osmolality can change over the course of the spawning season and with repeated stripping of milt (Munkittrick and Moccia 1987, Billard 1988, Aas et al. 1991, Lahnsteiner et al. 1997). Osmolality of seminal plasma of Atlantic salmon showed wide variation among 27 males ranging from 117 to 320 mOsmol/Kg (Aas et al. 1991). Fertilization was also variable, from 1 to 97%. Thus, there appears to be a number of assays that can be used to predict sperm quality.

The objectives of this study were to determine the utility of propidium iodide and carboxyfluorescein diacetate in the flow cytometry method for sperm quality determination in salmonids and to document the effects of osmolality and urine contamination on sperm quality in refrigerated and cryopreserved samples.

Materials and Methods

Quantitative data on the fluorescent-stained sperm were collected using an EPICS XL flow cytometer (Coulter Corporation Inc., Hialeah, Florida). The side-scatter and forward-scatter parameters were gated so that only those cells possessing the light scattering characteristics of sperm were analyzed for fluorescence. For each sample a total of 10,000 sperm were analyzed for the log of their fluorescence. The green fluorescence (carboxyfluorescein diacetate; 5 μL of stock solution 1 mg/mL) used to monitor mitochondrial activity was collected through a 525-nm bandpass filter and the red fluorescence (propidium iodide; 5 μL of stock solution 1 mg/mL in DMSO) used to indicate the structural integrity of the outer membrane, was collected through a 635-nm bandpass filter. Seminal plasma osmolality was determined using an osmometer (Precision Systems, Natick, Massachusetts). Chloride was determined with chloride analyzer (Corning model 925, Medfield, Massachusetts) and sodium was measured by atomic absorption using the modified method of Willis (1960).

In the first experiment, milt from Atlantic salmon males was collected into one of four treatments: 1) stored as a thin layer with oxygen; 2) stored as a thin layer with nitrogen; 3) diluted (1 to 100) into Erdahl and Graham's media (Erdahl and Graham 1987), and 4) diluted (1 to 100) into Billard's media (Billard 1977). After 24 hr, milt from the four treatments was used to fertilize two replicate lots of 300 eggs. Eggs were pooled from the spawns of three females. The percent of viable cells was measured by flow cytometry. Fertilization rate was calculated by recording the percentage of eyed embryos.

Because data from the first experiment showed a wide variation in the milt osmolality and the osmolality of published media were variable (175 to 400 mOsmol/Kg), the sec experiment focused on the effect of osmolality on sperm viability. In this experiment, four cryopreservation media were prepared: that of Mounib (1978), DCH Media (15 mM mannitol,

45 mM sucrose, 100 mM $KHCO_3$, 6 mM KCl, 10 mM lecithin, 6.8 mM ascorbic acid, 6.5 mM glutathione, 5% BSA, 10% egg yolk, and 10% DMSO), that of Stoss and Holtz (1983) and that of Legendre and Billard (1980). A double-strength recipe of each media was diluted to yield four solutions with osmolalities of 175, 230, 275 and 400 mOsmol/Kg. The osmolalities chosen spanned the range found when published media were prepared and the two intermediate values were chosen to bracket the expected osmolality of the collected milt. Milt from five steelhead trout were pooled and extended into each of the 16 media (1 part milt to 3 parts media). Diluted milt was placed into 0.5-mL French straws, put on dry ice for 15 min to freeze and were stored in liquid nitrogen (LN_2). Samples were thawed at 25 °C and were immediately diluted into 4.5 mL of either 300 or 600 mOsmol/Kg Erdahl and Graham's media. The percentage of viable sperm was determined by flow cytometry.

In the third experiment, the osmolality of fresh undiluted milt from Atlantic salmon and lake trout was determined. Milt from individual lake trout was also diluted into Erdahl and Graham's media of 335 mOsmol/Kg. After 24 hr of cold storage with oxygen or in diluent, the percentage of viable sperm was determined using flow cytometry.

For the fourth experiment, a comparison of motile and non-motile sperm was conducted. Samples were collected from eight Atlantic salmon males with motile sperm and from 12 males with non-motile sperm. Seminal plasma was separated by centrifugation at 500-× gravity for 10 min and stored frozen for later analysis. Osmolality, and concentrations of sodium and chloride were determined. The percentage of viable sperm was determined after 24 hr of cold storage on oxygen or diluted (1/100) in Erdahl and Graham's media.

The fifth experiment examined the effect of urine contamination on sperm viability. The percentage of viable sperm was determined in the milt of six Atlantic salmon males 24 hr after being mixed with urine. Urine from Atlantic salmon was collected on the same d as the milt samples. The following urine to milt (vol:vol) ratios were prepared: 1:9; 2:8; 4:6 and 6:4. This was the equivalent of 10, 20, 40 and 60% urine contamination.

Experiments 1, 3, 4 and 5 were completely randomized designs. The design of Experiment 2 was a 4 x 4 x 2 (4 media types x 4 media osmolalities x 2 post-thaw osmolalities) factorial arrangement with two replicates. Correlation coefficient (Experiment 1) and regression analysis (Experiments 3 and 5), analysis of variance and means were determined with the Statistical Analysis System program (SAS 1994). Percentage data were transformed (arc-sine) for statistical analysis.

Results and Discussion

Determination of the percentage of viable sperm using carboxyfluorescein diacetate in combination with propidium iodide was highly correlated ($r = 0.95$, $P < 0.05$) with egg fertilization (Table 1). An earlier study (Schneider 1993) with carboxyfluorescein diacetate alone was not found to be predictive of sperm quality. Our data are consistent with studies using rhodamine 123 as an indicator of mitochondrial activity (Ogier de Baulny et al. 1997). Use of fluorescence microscopy will provide data similar to those of flow cytometry, but the interpretation of the results will be less accurate based on visual observation because only a limited number of sperm cells can be counted.

Table 1. Percent egg fertilization and percent viable sperm measured by flow cytometry for Atlantic salmon.

Storage treatment	Fertilization (%)	Viable sperm (%)
Oxygen	75	87
Nitrogen	59	45
Erdahl and Graham media	67	77
Billard media	1	3
Correlation	r = 0.95, $P < 0.05$	

The osmolality of milt from individual Atlantic salmon males ranged from 185 to 291 mOsmol/Kg (231 $\pm$ 38; mean $\pm$ SD). This raised a number of questions about the importance of milt osmolality and its effect on sperm storage in media or diluents prepared at a median osmolality for that species. Furthermore, in our early cryopreservation work when media were prepared according to published reports, measurement of osmolality resulted in a wide variation (175 to 450 mOsmol/Kg) among the media.

Experiment 2 (Figure 1) showed a significant 3-way (media x osmolalities x post-thaw osmolalities) interaction ($P < 0.05$). After thawing, 600 mOsmol/Kg resulted in an increase in the percentage of viable sperm in DCH media whereas the percentage of viable sperm decreased in the other three media. The percentage of viable sperm increased at 300 mOsmol/Kg after thawing, in the media of Stoss and Holtz (1983) as osmolality increased. The opposite was found in DCH media. The percentage of viable sperm was variable in the remaining two media. The highest percentages of viable sperm in Legendre and Billard (1980) media were found at 175 and 400 mOsmol/Kg, not at 230 and 275 mOsmol/Kg. Overall, it appears that DCH media supported a higher percentage of viable sperm. Little attention has been given to the importance of controlling osmolality in cryopreservation media and we feel that the inconsistent results found in the literature are in part related to variation in the osmolality of media and milt.

After thawing, sperm cells in 600 mOsmol/Kg DCH media responded differently than did cells in the other three media. The recipe for DCH media was based on media used in metabolism studies with isolated mitochondria (Honeyfield and Froseth 1991). Additional research is needed to explain these observations and to understand the effect of cellular uptake of media components and their cryogenetic properties when used with fish sperm.

High variability was observed in milt osmolality of lake trout (Figure 2a). As milt osmolality increased, the percentage of viable cells increased (Figure 2a). Furthermore, if these milt samples were diluted at collection into a media with an osmolality of 335 mOsmol/Kg, the percentage of viable sperm was improved (Figure 2b) for most samples. Also, the percentage of viable sperm was similar in diluted samples (Figure 2b) to that of sperm which were separated from seminal plasma, diluted within 30 min, and placed in cold storage for 24 hr with oxygen (data not shown). This suggests that the elements that contribute to seminal plasma osmolality and buffering capacity are important to sperm survival. Future research should focus on seminal plasma composition, its production and secretion and expand on work that has been conducted in this area (Scott and Baynes 1980, Munkittrick and Moccia 1987, Schmehl et al. 1987, Morisawa and Morisawa 1988, Gallis et al. 1991, Ciereszko and Dabrowski 1994, Ciereszko et al.1996).

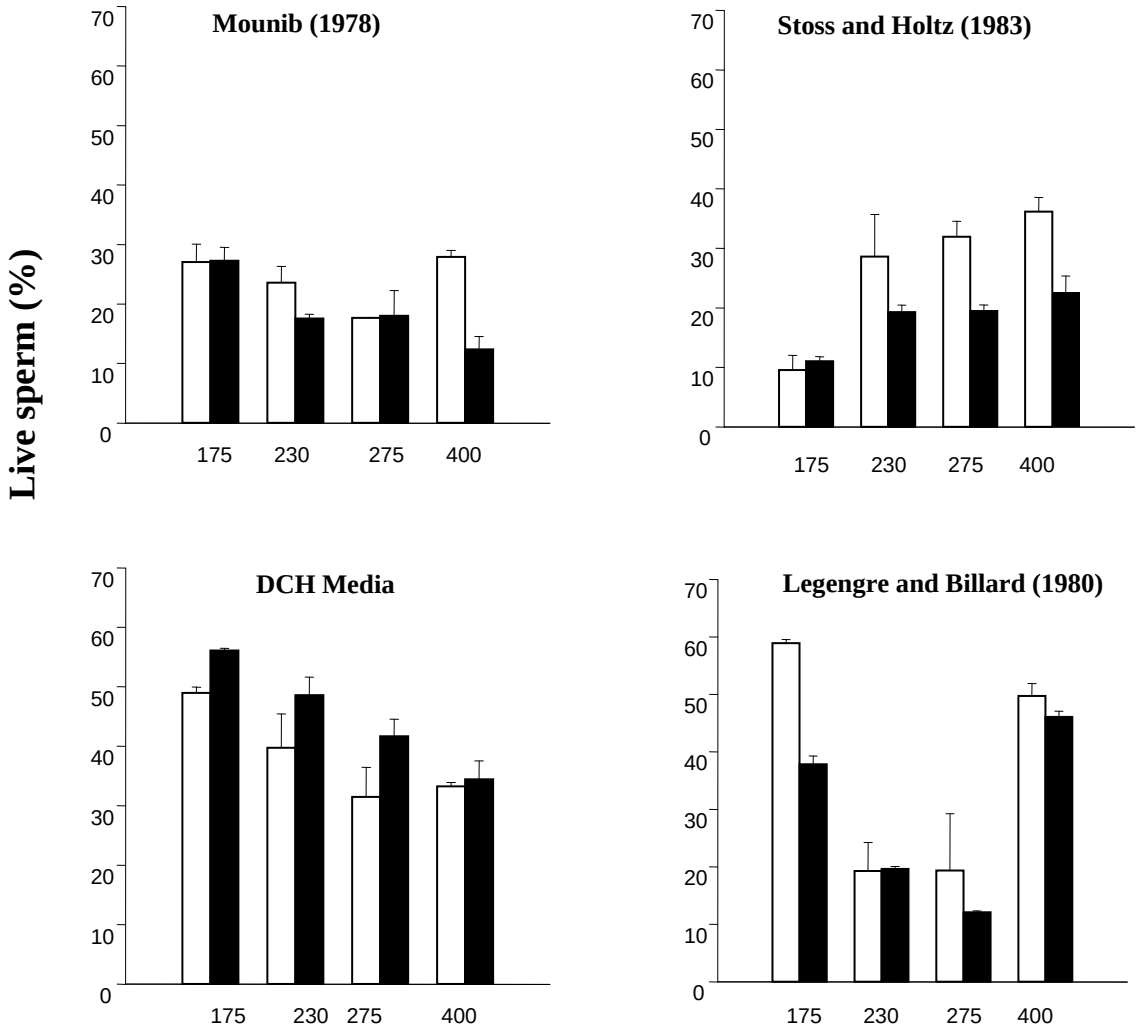

Figure 1. Effect of media, osmolality of the media and osmolity of thawing diluting media on percentage of viable sperm in cryopreserved milt of steelhead trout. Samples were diluted after thawing into solutions of 300 mOsmol/Kg (white bars) or 600 mOsmol/Kg (black bars).

There were differences in seminal plasma osmolality and sodium and chloride concentrations between motile and non-motile sperm of Atlantic salmon ($P < 0.05$) (Table 2). Values for all three variables were lower in non-motile samples. Placing non-motile sperm into a diluent at collection made no difference in the percentage of viable cells observed. Therefore if visual inspection indicated that sperm were not motile, placing them into diluent did not revive them.

Osmolality is a critical variable in sperm quality. It is suggested that osmolality of media and diluents always be measured before use. As seen in our studies and those of others (Aas et al. 1991), milt osmolality among males is highly variable. We recommend that when sperm are to be stored, collecting milt into an iso-osmotic diluent will improve sperm survival.

Sperm motility has been widely used to predict sperm quality. Measurement of motility can be accomplished using simple microscopy or by computer-assisted analysis. The benefit of the microscopy method is that it is rapid and can be conducted where fish are cultured. However, this method is highly subjective and depends upon a trained individual. In salmonid species, sperm are motile for only 10 to 60 sec. Visually, it is relatively easy to categorize cells with 90 to 100% or 0 to 10% motility, but it is more difficult to accurately assign motility values of between 20 to 80%. Yet this method allows one to discard sperm samples with little or no motility. Computer analysis of sperm motility recorded with a high-speed video camera resulted in a more objective determination of motility and is predictive of sperm viability (Lahnsteiner et al. 1996, Toth et al. 1997). Information available using this technique includes percentage of motile cells, types of movement (circular, linear and non-linear) and sperm velocity. Computer-assisted analysis and flow cytometry are each excellent methods for evaluating sperm quality, although instrumentation costs and availability may limit their use.

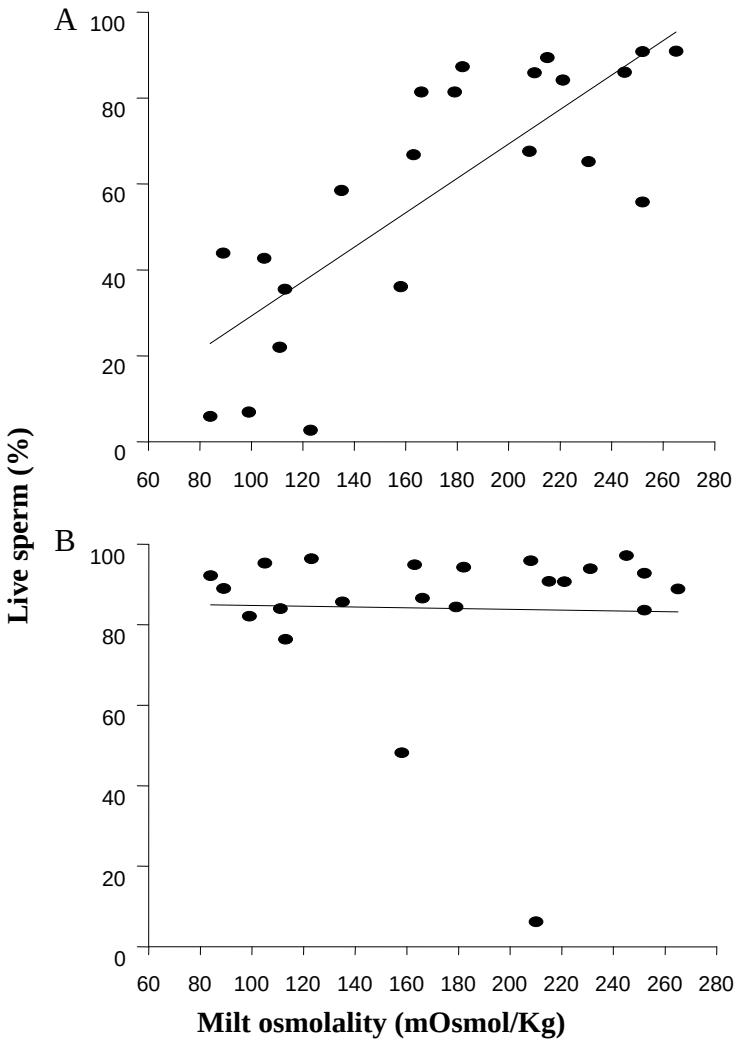

Figure 2. Osmolality and percentage of viable sperm (A) from lake trout after 24 hr of storage with oxygen (-10.77 + 0.4x; $r^2 = 0.63$) and percentage of viable sperm (B) in milt diluted into 335 mOsmol/Kg media (Erdahl and Graham 1987) at time of collection and measured after 24 hr of storage ($r^2 = 0.001$).

Table 2. Values (mean ±SE) for seminal plasma measurements from Atlantic salmon milt.

	Number	Osmolality (mOsmol/Kg)	Chloride (mEq/L)	Sodium (mEq/L)
Motile sperm	8	209 ± 17	86 ± 6	78 ± 7
Non-motile sperm	12	162 ± 14	64 ± 6	54 ± 5

Urine contamination of Atlantic salmon milt reduced the percentage of live sperm cells in all samples (Figure 3). However, in four samples (Figure 3a) the slope of the lines indicated that urine reduced sperm viability by only 0.6 to 2.5% per unit of urine contamination, whereas in the remaining two males (Figure 3b) a more dramatic decrease was observed (5.6 to 9.2% decrease per unit of urine). Based on this study, urine contamination in salmonids does not appear to be as detrimental as that observed in common carp *Cyprinus carpio* (Perchec et al. 1995).

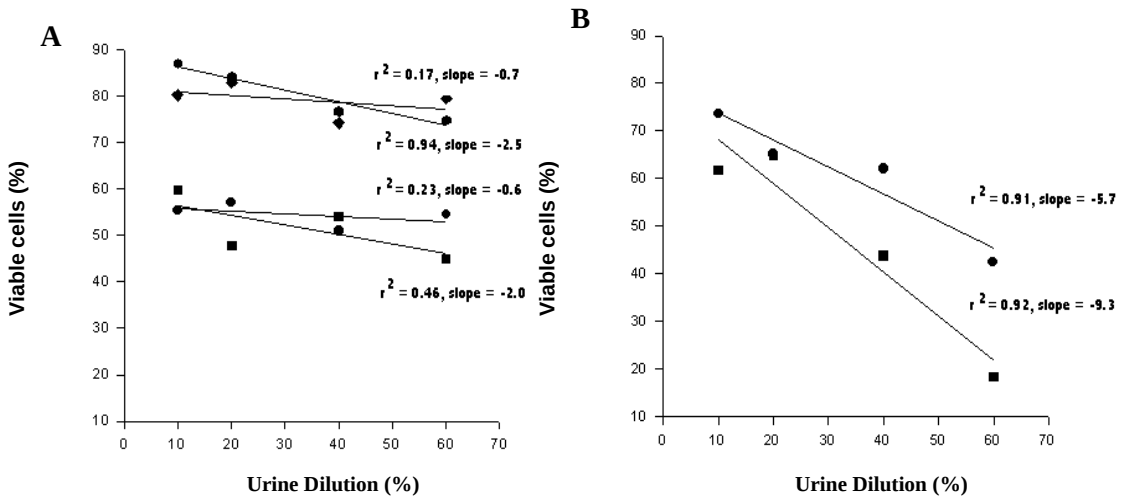

Figure 3. The effect of urine dilution on percentage of viable sperm in six individual Atlantic salmon males. Panels show the four males (A) least affected and the two males (B) most affected by urine dilution.

In conclusion, our data and those of others (e.g. Lahnsteiner et al. 1996) support the use of osmolality or ion concentrations in concert with motility determination as the minimum criteria for evaluating collected milt. Flow cytometry or computer-assisted motility analysis are more objective and precise, but instrumentation costs may limit their use.

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Annotated Bibliography of Developments in the Last Decade

Assessment of milt quality and factors affecting viability of fish spermatozoa has been an active field, and a continual point of interest in the field of cryobiology. Technologies for assessing fish sperm morphology, viability, DNA integrity, quality were increasingly applied to aquatic sperm quality assessments since 2000. Sperm quality assessment techniques include staining techniques for evaluating membrane and organelle integrity, including flow cytometry,

Annexin V-Flourescein staining, phase contrast and epiflourescent microscopy, image cytometry, and other techiques such as electrophoresis (comet assay) and computer assisted sperm analysis (CASA). Further, this edition of the book incorporates a new section titled "Gamete Quality," including new chapters on sperm quality evaluation, flow cytometry applications in mammals and aquatic species, and a review of CASA.

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New Chapter

Sperm Quality Assessment in Mammals by Flow Cytometry

Phillip Purdy

Introduction

The fertility of a semen sample cannot be predicted, whereas the potential for poor fertility can be estimated by evaluating physiologic and morphologic factors (Amann and Hammerstedt 2002). This rationale is based on the idea that while a significant amount of information is known about sperm function, greater still is the unknown information, including the mechanisms that are required for achieving successful fertilization. Because of this lack of knowledge, the ability to evaluate multiple sperm characteristics simultaneously is why flow cytometry can be of tremendous value. This chapter will provide an overview of flow cytometry, and will include descriptions of standard procedures used to evaluate mammalian sperm that may be adapted for use in sperm from aquatic species. In addition, the concept of multiplexing will be introduced, providing applications that offer potential for varied analyses of sperm quality and fertilizing potential.

Mechanics of Flow Cytometry

Flow cytometry enables evaluation of a large number of cells (usually 5,000-50,000) in less than 1 min. The sample stream is introduced into the instrument such that single cells align within a sheath fluid (clear analysis medium that suspends particles for analysis). The sheath fluid passes through a chamber where the fluorescent stains used to label the target cells are excited with one or more lasers. The number of lasers is determined by the fluorescence excitation wavelength necessary for each stain. For example, fluorescein isothiocyanate (FITC) requires a 488-nm laser for excitation, but if a second stain requiring a different wavelength is added, a second laser will be needed. Initially the sperm are detected based on the forward and side light scatter, which are indicators of the size and granularity of cells. Fluorescence emission is detected using a series of photomultiplier tubes (PMT) so that the percentages of cells or the amount of fluorescence in populations of interest can be determined. As an example, sperm plasma membrane integrity is commonly evaluated (Figure 1, next page). To do this, a combination of SYBR 14 and propidium iodide (PI) is used to distinguish target cells (SYBR 14 positive) from debris (non-fluorescing particles of a similar size), and to determine which of these cells have damaged membranes (PI positive) (Garner et al. 1994). In some flow cytometers, the emitted fluorescence passes through a long-pass filter (e.g., >515 nm) to remove background light (laser) from analysis. The fluorescence encounters one or more dichroic mirrors that reflect light via optical filters to the PMT for detection of different fluorescence wavelengths. Fluorescence from SYBR 14 is typically detected through a band-pass filter in the region of 525 nm, while PI fluorescence is often detected through a long-pass filter at wavelengths greater than 630 nm. A band-pass filter only allows fluorescence within a certain wavelength range to pass, whereas a long-pass filter only allows fluorescence above a certain wavelength to pass.

Purdy, P. 2011. Sperm Quality Assessment in Mammals by Flow Cytometry. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 219-226.

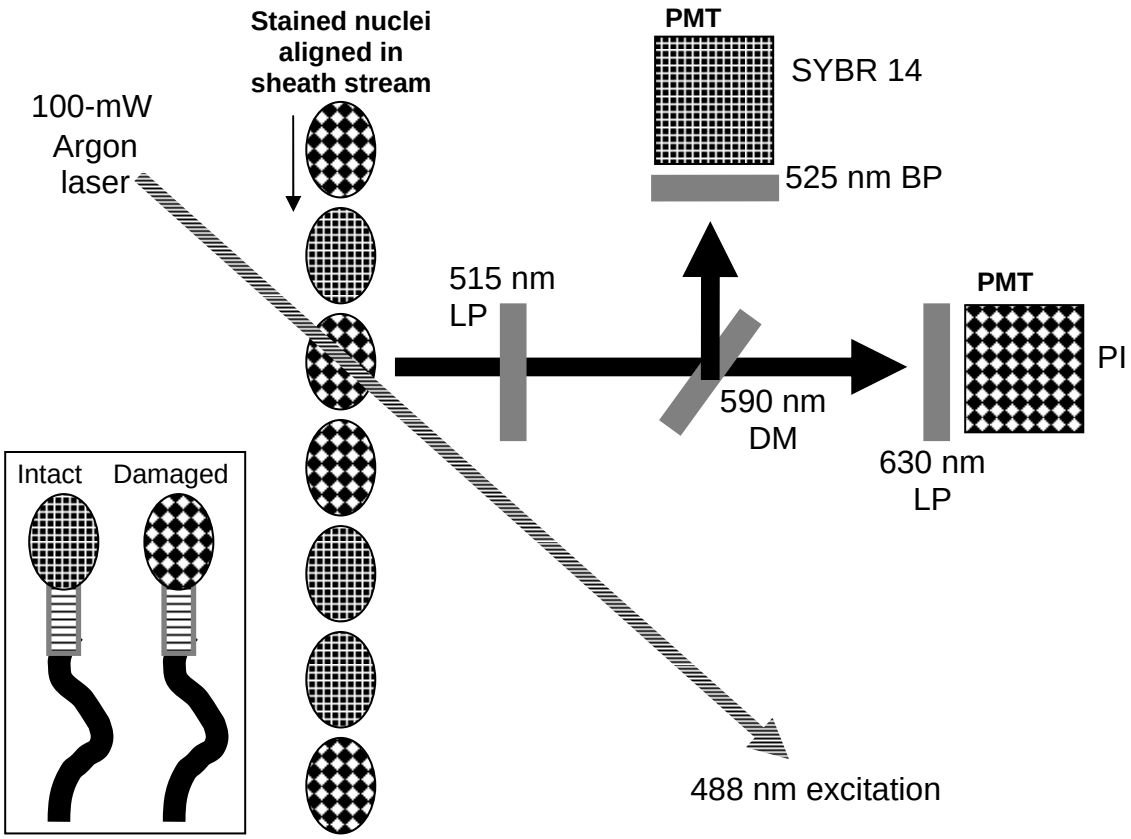

Figure 1. Schematic representation of the flow cytometric evaluation of sperm for intact plasma membranes (SYBR 14 positive) and compromised membranes (propidium iodide (PI) positive). The analysis requires the use of long-pass (LP) and band-pass (BP) filters as well as a dichroic mirror (DM) for detection of stained sperm by specific photomultiplier tubes (PMT).

Flow Cytometry Applications

The SYBR 14/ PI analysis is one specific example of the use of flow cytometry. One of the advantages of flow cytometry is that once a staining methodology is mastered, the same principles can be applied to other fluorescent probes. In the previous example the fluorescence emission was 525 nm for SYBR 14, and >630 nm for PI. After the flow cytometer is set up to analyze these stains, it is easy to substitute and perform different analyses as long as the fluorescence excitation and emission wavelengths remain the same. The only differences may be in the manner in which the cells are prepared for staining. This is performed on a routine basis in mammalian and aquatic species analyses. Another commonly used stain is rhodamine 123 (R123), which is used to evaluate mitochondrial membrane potential. R123 and SYBR 14 are both excited at 488 nm and emit at 525 nm so each can therefore be counter-stained with PI to identify sperm with intact plasma membranes, as has been done for Nile tilapia *Oreochromis niloticus* (Segovia et al. 2000) eastern oyster *Crassostrea virginica* (Paniagua-Chávez et al. 2006) red seabream *Pagrus major* (Liu et al. 2007) and three species of sturgeon (Horváth et al. 2008). Other stains with similar excitation and emission spectra can also be interchanged, for example the calcium indicator Fluo-3AM (Morita et al. 2003, Purdy et al. 2007) or Annexin V which is an indicator of membrane quality and apoptosis (Purdy et al. 2007, Beirão et al. 2008).

These reports demonstrate an important point: if the stains can be used with sperm and visualized with fluorescence microscopy (e.g., Morita et al. 2003, Beirão et al. 2008), they can generally be adapted for use with flow cytometry (Purdy et al. 2007).

Additional Staining Techniques

In addition to R123, mitochondrial membrane potential can also be detected using other stains such as MitoTracker Green FM (MITO) (Haugland et al. 1996) or JC-1, which provides a more detailed assessment of mitochondrial function (Garner et al. 1997). In this instance the fluorescence excitation and emissions are the same as for R123, so JC-1 can be counter-stained with PI for detection of membrane integrity. The advantage of using JC-1 over R123 or MITO is that JC-1 will reveal distinct populations of sperm with low, high, or intermediate mitochondrial activity whereas R123 and MITO are only able to reliably detect a single (qualitative) population of cells (Garner et al. 1997).

Other staining combinations can also be performed using flow cytometry, such as analysis of nuclear DNA content (e.g., Tiersch et al. 1989), chromatin integrity by sperm chromatin structure analysis (SCSA) (Evenson et al. 1980), or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Li et al. 2003, Oosterhuis et al. 2000, Anzar et al. 2002) but this may require different lasers or filter combinations depending on the fluorescent spectrum of the probes that are used. For example, the use of acridine orange for detection of chromatin decondensation requires excitation at 488 nm and emission detection at 525 nm (for double-stranded DNA) and at 620 nm (single-stranded DNA or RNA) (Evenson et al. 1980). Regardless, the end result is the same enabling identification of populations of sperm with decondensed or degenerated chromatin.

Two more detailed assays that analyze the plasma membrane of the cells have been performed with mammalian sperm to better understand cryopreservation and evaluate quality of sperm samples. To study membrane fluidity (a measure of membrane lipid organization) of mammalian sperm, investigators have used merocyanine 540 (M540) (Flesch et al. 2001, Hallap et al. 2006, Marchiani et al. 2007) or N-((4-(6-phenyl-1,3,5-hexatrienyl)phenyl) propyl)trimethylammonium-p-toluenesulfonate (TMAP) (Purdy et al. 2005). Both protocols counter-stain sperm with PI, but M540 excites at 488 nm and emits at 525 nm (Gadella and Harrison 2000), while TMAP excites at 355 nm and emits at 525nm (Fox and Delohery 1987). Fluidity assays can be used to examine the membrane status of mammalian sperm, and can be used to determine changes in phospholipids due to capacitation (Flesch et al. 2001), environmental effects (e.g., cold shock) (Hallap et al. 2006), or different levels of epididymal maturation (Gadella and Harrison 2000, Flesch et al. 2001) or quality (Marchiani et al. 2007). Furthermore, this assay can also examine how membrane modifications (e.g., cholesterol addition or removal) can affect sperm prior to and during the cryopreservation process (Purdy et al. 2005).

Another useful assay is used to investigate the ubiquitination of mammalian sperm for determination of fertilizing potential (Lovercamp et al. 2007, Sutovsky et al. 2002, 2003). The rationale for this assay is that apoptotic sperm will be labeled with the ubiquitin protein so that the sperm can be phagocytized within the testis or the epididymis (Sutovsky 2003). Consequently, sperm samples with a high percentage of ubiquitinated sperm have been correlated with lower fertility (Lovercamp et al. 2007, Sutovsky et al. 2002, 2003). This assay

appears best used in conjunction with other assays to provide a broad analysis of sample quality (Purdy 2008).

A further example of the evaluation of membrane quality and apoptotic-like events is the use of Annexin V. This fluorescent probe binds to phosphatidlyserine (PS) on the outer membrane of damaged sperm (Glander and Schaller 1999, Anzar et al. 2002). Healthy mammalian sperm only have PS on the inner surface of the plasma membrane, but damage due to cryopreservation (Müller et al. 1999, Anzar et al. 2002) or other events can cause translocation of PS to the outer surface where it is readily labeled with Annexin V. Thus, samples with large percentages of sperm positive for Annexin V are considered to be of inferior quality in human, bull (Anzar et al. 2002), and ram (Glander and Schaller 1999, Müller et al. 1999). Similarly, in rainbow trout *Oncorhynchus mykiss* a preliminary study using Annexin V and PI found that samples of thawed milt that activated readily and had exposed PS were of lower quality, and after insemination yielded lower percentages of fertilized eggs (Purdy et al. 2007).

It is also possible to evaluate processes occurring within mammalian sperm. As mentioned above, calcium measured using Fluo-3 AM (Bailey and Storey 1994, Parrish et al. 1999, Marquez and Suarez 2007, Colás et al. 2009, Kardirvel et al. 2009) or Indo-1 (Mendoza and Tesarik, 1993, Dubé et al. 2003) can be monitored and used to identify the capacitation potential of a sperm sample (Parrish et al. 1999, 2004, de Vries et al. 2003, Purdy and Graham 2004). Furthermore, while calcium levels are an indicator of capacitation and the acrosome reaction, the intracellular signaling events of these processes can be modulated and thus monitored using a combination of calcium and PS staining assays (de Vries et al. 2003).

Multiplexing of Stains and the Future of Analyses

Up to this point the examples have been limited to counterstaining methodology, meaning a specific sperm characteristic (such as mitochondrial activity) is analyzed (stained), and the sperm are counter-stained with a viability indicator such as PI. These analyses provide useful data but the methodologies are not efficient for several reasons. Multiple aliquots per sperm sample must be prepared to analyze different sperm characteristics and depending on the instrument used, the flow cytometer may also have to be re-calibrated due to the different staining procedures or different desired laser settings for each stain. For example, although SYBR 14 and Annexin V excite and emit at the same wavelengths, it may be necessary to use different voltage settings on the laser to excite the specific stains (Purdy, unpublished data). As a result, the user may have to analyze all of the samples for a particular stain, and then recalibrate the flow cytometer for the new stain before analysis of the second set of samples. The drawback can be degradation of the samples while waiting, particularly if thawed sperm are being analyzed, which can produce incongruous results due to sample status and quality. This is more of an issue with older flow cytometers because the newer systems enable the user to rapidly switch analysis protocols by use of software and maintain the integrity of each analysis.

Until recently, the ability to combine stains for multiplexing (staining and analysis using three or more stains) has been an issue primarily due to a limited number of fluorescent stains. Companies such as Molecular Probes/Invitrogen (Eugene, Oregon, USA) and others have addressed this issue, by producing new stain combinations that incorporate a variety of excitation and emission wavelengths. Peanut lectin (PNA), for example, has been classically used in mammalian species for identifying acrosome-reacted sperm (Tao et al. 1993, Cheng et al. 1996, Nagy et al. 2003, Purdy 2008). Because the most common laser setting was 488 nm the PNA

lectin was conjugated with a FITC molecule to emit at 525 nm. As a result FITC-PNA could not be combined with most other probes for simultaneous evaluation of sperm characteristics, other than PI for membrane integrity. To overcome this issue, the PNA lectin can now also be purchased from Invitrogen with the conjugated fluorescent molecules ALEXA 488, 568, 594 and 647 nm (emission values), thus enabling combination with other fluorescent stains for simultaneous analysis within a single sample. Several companies are now producing probes that can fluoresce at multiple wavelengths similar to the range of ALEXA molecules. While the choice of fluorescent stains for any application is limited by the lasers and filters of a particular flow cytometer, the expansion of products provides a greater range of applications for most flow cytometry systems.

Many flow cytometers are now equipped with two or three lasers and numerous filter sets (e.g., seven on the Cyan ADP model, Beckman Coulter, Miami, Florida, USA) enabling simultaneous evaluation of multiple cell characteristics. For example, simultaneous analysis of SYBR 14, phycoerythrin-PNA, and PI can be used to identify the sperm population (SYBR 14 positive) while excluding other particles such as egg yolk (no fluorescence/SYBR 14 negative), sperm with intact acrosomes (PE-PNA negative), and sperm with intact plasma membranes (PI negative), all within individual samples (Nagy et al. 2003, Hallap et al. 2006). Furthermore, this analysis can also provide data about live, acrosome-reacted populations of sperm (SYBR 14 positive, PE-PNA positive, PI negative) and dead populations (PI positive). As a result, the time-consuming labor of staining and standardization for multiple sample preparations is minimized. The real value is that researchers can evaluate multiple characteristics in the same sample and thus avoid tube-to-tube variation in physiologic evaluations, all within a matter of sec.

Conclusions

The goal of flow cytometric evaluation of sperm from any species is to reliably analyze a large number of cells for multiple physiological characteristics in a short time. The use of dual-staining and multiplex analyses enables this, however flow cytometric evaluation should be used in combination with other analyses, such as microscopic or automated motility evaluation. As a result, a more accurate picture of the quality or physiological state of sperm samples will be determined and therefore provide greater feedback to investigators or other end users. Information of this sort on gamete quality will assist commercial adoption and widespread application of cryopreservation in aquatic species.

Acknowledgements

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New Chapter

Male Germplasm in Relation to Environmental Conditions: Synoptic Focus on DNA

Jill A. Jenkins

Introduction

Wild animals are generally more sensitive than humans to environmental stressors, thus they can act as sentinels for resource degradation. Sublethal stress is generally manifested first at the sub-organismal level, where immune systems are compromised, reproductive success is reduced, and genetic integrity is altered. Biomarkers – variables quantifiably responsive to changes in the environment – provide useful information to resource managers and regulatory agencies. Biomarkers of sperm quality are proving useful in this capacity, as well as in artificial breeding. Cellular and molecular bioassays can help to determine mechanisms of action of deleterious agents, predict fertility and reproductive potential, and model population-wide and community level effects. A sequence of biomarker assays can be tailored to fit species of concern, to study physiological effects responsive to known contamination events, and can be selectively applied to fresh, thawed, and fixed samples, as well as those shipped to the laboratory from field sites (Jenkins et al. 2010).

Prognostic sperm quality criteria of the World Health Organization (WHO 1987) include motility, cell morphology, and sperm counts. Other andrology endpoints can include evaluation of sperm membrane integrity (viability), mitochondrial membrane potential (mitochondrial function), acrosome reactivity, and chromatin condition. Because particular parameters provide variable utility in studies (Marchetti et al. 2002, Malo et al. 2005) and are not often or consistently evaluated, this chapter will focus on the utility of sperm DNA. Structural integrity of DNA can yield superior diagnostic and prognostic information on fertility potential (Agarwal and Said 2003), is particularly important for fishes that reproduce by external fertilization, and has been suggested for use as a primary measure in examining among-male variation and for choosing samples for further study (Jenkins et al. 2011). Sperm chromatin condition can be measured by a variety of techniques for a multitude of endpoints.

The Field Investigation Approach

Characterizing reproductive condition of fishes from site locations typically begins with whole animal condition, organosomatic indices (gonadosomatic index [GSI] and hepatosomatic index [HSI]) (Schmitt et al. 1999). For collection of wild fish for endocrine disruption studies, a few geographically extensive surveys have focused on subsets of biomarker assays for potential endocrine disruption, such as determination of plasma sex-steroid hormones, stage of gonadal development, and plasma vitellogenin, which is an egg protein that indicates exposure to estrogenic compounds when found in male fish (Goodbred et al. 1997). Values for HSI can decrease in fish stressed by adverse changes in water quality (Lee et al. 1983), and sometimes reflect fish nutritional state because of liver glycogen storage, yet HSI can increase after exposure to certain types of contaminants (e.g., petroleum hydrocarbons) (Baumann et al. 1991). However,

Jenkins, J. 2011. Male Germplasm in Relation to Environmental Conditions: Synoptic Focus on DNA. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch. and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 227-239.

use of cell and molecular biomarkers lessens the uncertainty of data interpretation in investigations involving endocrine disrupting compounds (EDC). As stated above, prognostic sperm quality criteria can include evaluation of motility, cell morphology, sperm counts, sperm membrane integrity (viability), mitochondrial membrane potential (mitochondrial function), acrosome reactivity, and chromatin condition. Structural integrity of DNA can yield superior diagnostic and prognostic information on fertility potential, and sperm chromatin condition can be measured by a variety of techniques and endpoints. Studies of this type are ideally suited for application of flow cytometry to evaluate single cells such as from sperm or blood, or cell suspensions derived from tissues, and have been used for a variety of applications. Evaluation of spermatogenesis illustrates of the breadth of application for flow cytometry (Table 1).

Table 1. Representative studies using flow cytometry for assessment of spermatogenesis.

Discipline	Model	Citation
Assisted reproduction, dysfunction diagnosis	Human	Kaufman and Nagler 1987, Giwercman et al. 1994, Dey et al. 2000, Coskun et al. 2002, Levek-Motola et al. 2005
Chemotherapeutic testing	Mouse	Jyothi et al. 2001
	Hamster	Weissenberg et al. 2002
Toxicity testing	Rat	Wade et al. 2006, Yoon et al. 2003
	Mouse	de Vita et al. 1995, Evenson et al. 1986
Reproductive biology	Mouse	Petit et al. 1995
	Rat	Malkov et al. 1998, Suter et al. 1997
	Mammals	Suresh et al. 1992
	Tilapia	Tokalov and Gutzeit 2005
	Loach	Yoshikawa et al. 2009
	Medaka	Hong et al. 2004
	Eel	Miura et al. 2002
Endocrine disruption	Mosquitofish	Jenkins et al. 2009, Jenkins and Draugelis-Dale 2006

Other than some studies with captive male broodstock and artificial spawning efforts, few investigations have addressed evaluation of sperm quality by flow cytometry in natural populations of fish or mammals (Jobling 2002a,b, Jenkins and Goodbred 2005, Malo et al. 2005, Jenkins and Draugelis-Dale 2006, Goodbred et al. 2007, Marr 2007, Jenkins et al. 2011). However, strategies for field collection and laboratory assessments of sperm quality were recently developed for use with endangered razorback suckers *Xyrauchen texanus* (Jenkins et al. 2011), where field collections were coupled with laboratory analyses more than 1,600 km distant. Enhanced success in field collection of milt or testes translates to meaningful analyses and data interpretation, where the requirements for each species include specific ionic, osmotic, and pH conditions, and use of antibiotics (e.g., largescale suckers *Catostomus macrocheilus* from the Columbia River Basin).

Endocrine Disruption

Hormonally active EDC can alter function of the endocrine system to cause adverse effects in organisms, progeny, or subpopulations (Vos et al. 2000). Agricultural runoff, human wastewater effluent, and industrial sources including inorganic and organic chemicals introduce mixtures of estrogenic and androgenic compounds into the aquatic environment (Kolpin et al.

2004, Orlando et al. 2004). Feral male fish have been illustrative of site-specific ECD effects, where common carp *Cyprinus carpio* in Lake Mead National Recreation Area, Nevada/Arizona displayed lower gonadosomatic indices, lower proportions of sperm relative to other germ cell stages, higher incidences of macrophage aggregates (Patiño et al. 2003), and other reproductive parameters (Goodbred et al. 2007, Jenkins and Goodbred 2005). Within the Potomac River where notable fish kills have occurred, male smallmouth bass *Micropterus dolomieu* showed high incidences of microscopic testicular oocytes, indicating exposure to EDCs (Blazer et al. 2007). Because the reproductive physiology of vertebrates is similar, cross-taxa comparisons are relevant (Mills and Chichester 2005), hence enabling comparisons across species.

When the male endocrine system is affected, the function of spermatozoa may be influenced. Wild male roach *Rutilus rutilus* receiving treated sewage effluents in rivers in the United Kingdom were more affected than females, displaying reduced milt volume and sperm density, and motility and fertilization were negatively correlated with histological feminization (Jobling et al. 2002a,b). Some EDC congeners and metabolites are known to have specific targets, such as di-*n*-phthalate which produces adverse effects on the male reproductive tract, ultimately disrupting spermatogenesis (Lee and Veeramachaneni 2005), and polychlorinated biphenyls which interfere with androgen receptor ligand-binding domains (Portigal et al. 2002) and reduce human sperm counts (Rozati et al. 2000).

Additionally, many EDC are considered to be toxic, or as having carcinogenic potential, adding substantially to adverse health effects in light of the mutagenic properties relating to the EDC itself and the oxidative damage to DNA generated by compound metabolism or inflammation (Sϕrensen et al. 2003). For example, hexachlorobenzene (HCB), a fungicide banned for use in the United States since the mid-1960s, is considered an animal carcinogen. Normal development of the male rat reproductive tract has been impeded by HCB, which interferes with androgen action (Ralph et al. 2003). Taken together, male germplasm represents an ideal cell and molecular target for in-depth studies of potential effects of compromised aquatic conditions. The remaining text will focus on technologies, in particular flow cytometry, that are often used in assessments of male fertility in humans and wild animals that can be adapted for use with species of interest.

Spermatogenic Stage of Cellular Development

The progression of spermatogenesis in fish is influenced by estrogenic, androgenic, and progestogenic sex steroids (Schulz et al. 2010). This highly organized and coordinated process is characterized by mitotic and meiotic divisions that transform the spermatogonia into final, mature genome vectors, the spermatozoa (Schulz et al. 2010). While fishes vary in the number of spermatogonial generations, the process is conserved in vertebrates. Spermatids undergo a final differentiation period known as spermiogenesis, during which the DNA is maximally compacted. Chromatin is a complex of DNA, histones, and non-histone proteins, whereby during spermiogenesis protamines with a high charge density replace histones resulting in maximal genome compaction. Because these small nuclear basic protein types vary among fishes (Ausió et al. 2007), their genome compaction level is variable. Because ratios among protamines and histones are important determinants of fertility for mammals, this warrants further investigation in fishes, especially in light of cryopreservation of sperm from endangered species (Ausió et al. 2007).

The sex steroids are mainly produced in the gonad, and the endocrine system plays a critical regulatory role in the early developmental stages of spermatogenesis. In fish, higher proportions of spermatids reflect greater spawning readiness, and generally a higher gonadosomatic index. Within spermatogenic cells, the relative numbers of nuclei with different ploidy levels are easily measured by DNA staining followed by analysis using flow cytometry (Figure 1, next page). This process is less subjective and time-consuming than histopathological or stereological methods.

Flow cytometry is a sophisticated way to reveal spermatogenic processes and has long been used with humans as a non-invasive clinical assay and with animals in various studies, but has only been applied sparingly for field EDC studies for which histopathology data exist (Aravindakshan et al. 2004, Lee and Veeramachaneni 2005). Flow cytometric data were indicative of site differences in a study along a gradient of wastewater treatment plants where the western mosquitofish *Gambusia affinis* was a surrogate fish model for the federally listed Santa Ana sucker *Catostomus santaanae*. This type of detailed information (Figure 1, next page) can be of utility in monitoring and regulating the presence of EDC in waterbodies, as well as in aquaculture biotechology applications (Schulz et al. 2010).

Staining of DNA in conjunction with other cell parameters such as cell size, granularity, mitochondria, and proteins has allowed for differentiation of somatic cells in rat testicular cell suspensions (Suter et al. 1997, Malkov et al. 1998), in primary germ cell culture of Nile tilapia *Oreochromis niloticus* (Tokalov and Gutzeit 2005), and in the unisexal loach *Misgurnus anguillicaudatus* (Yoshikawa et al. 2009). Overall, different phases of the cell cycle can be accurately quantified by flow cytometry (Dallas and Evans 1990). For example, if diploid white blood cell types are inherent in milt samples, this may indicate an immunologically challenged individual.

Sperm Counts

Sperm counts are a fundamentally important predictor of male fertility, and knowledge of numbers *in vitro* is a critical quality control measure for cryopreservation studies (Dong et al. 2007). Although fairly well studied with rodent models, few studies have been conducted in aquatic species to examine whether estrogenic chemicals affect the numbers of sperm produced. Reduced testosterone in western mosquitofish could contribute to lowered sperm counts (Toft et al. 2003). In guppies *Poecilia reticulata,* sperm counts were dramatically reduced following 21 d of exposure to low levels of tributyltin or bisphenol A (Haubruge et al. 2000), and after feeding with anti-androgenic compounds *p,p'*-DDE, a metabolite of the banned pesticide dichloro-diphenyl-trichloroethane(DDT), and the fungicide vinclozolin, yet GSI was not affected in experiments with guppies (Bayley et al. 2002). These results underscore that cellular responses are sensitive indicators of reproductive quality, and that thresholds vary among species.

Many techniques and instruments can be used for estimating numbers or density of spermatozoa per unit volume of milt or weight of testis, including hemocytometers, spermatocrit, Coulter counter, and spectrophotometry. Flow cytometric counting can be performed in several ways using different methods and instruments, and provides accurate assessments (Evenson et al. 1993, Eustache et al. 2001). In relation to DNA, sperm counts can be performed by staining nucleic acids and comparing these values to a known number of fluorospheres (Jenkins et al. 2011).

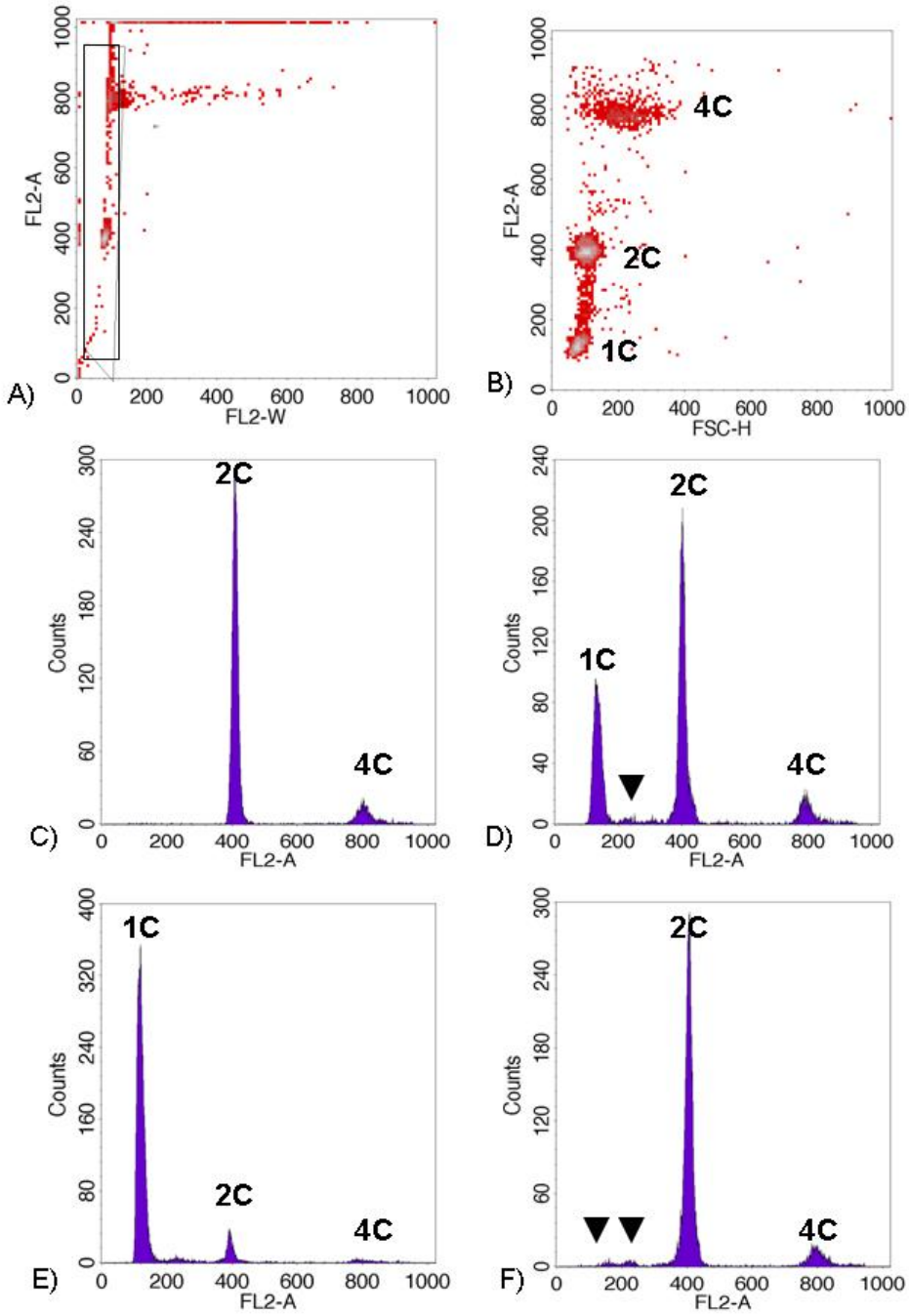

Figure 1. Flow cytometric histograms of western mosquitofish testicular cells stained with propidium iodide to analyze relative DNA content. A) Only single nuclei were evaluated (those within the rectangular gate). Aggregates of nuclei were excluded from analysis by using "doublet discrimination" based on width (FL2-W) and area (FL2-A). B) The "1C" (haploid) peak included spermatids and spermatozoa; "2C" included spermatogonia, somatic cells, and secondary spermatocytes, and "4C" included primary spermatocytes and somatic cells at the mitotic (G2/M) phase. Data from the same individual are shown in panels A and C, from another individual in B and D, and two other individuals in E and F. In panels C-F), peak heights indicate varied proportions of haploid, diploid interphase (2C), and post-DNA-synthesis diploid (4C) nuclei, and arrowheads indicate haploid nuclei at two levels of DNA compaction.

Integrity of DNA

The potential for drugs and other agents to induce cytotoxic or genotoxic cellular effects has been measured using flow cytometry (Dallas and Evans 1990). Clastogenic agents cause a loss of DNA integrity, where broadening of the G_0-G_1 (interphase) peak in cell populations exposed to cytotoxic agents is a biomarker response (Dallas and Evans 1990). Fragmentation of nuclear DNA is a biomarker that has been related to effects in individuals and populations (Figure 2, next page). A limited number of agents damage DNA, including radiation (Blaustein et al. 1997), reactive oxygen intermediates that are generated in excess from decontamination processes (Shen and Ong 2000), and direct contact with environmental anthropogenic chemicals. Such factors, and a lack of antioxidants, can interact to damage DNA (Lopes et al. 1998). Loss of DNA integrity, induction of DNA repair proteins, and apoptosis (also characterized by loss of DNA integrity) are mechanistically linked, occur somewhat sequentially kinetically, and their measurement provides an integrated and comprehensive assessment of the cytogenetic process.

Genomic DNA alterations or fragmentations are widely used in physiological, genetic and toxicological studies. Many waterborne contaminants have cytogenetic properties, which cause enhanced frequency of chromosomal aberrations or the alteration of the structure of DNA. Field studies documenting altered blood DNA content or profile upon exposure have included radionucleotides with turtles and ducks (George et al. 1991), mercury with largemouth bass (Sugg et al. 1995), petrochemicals with wild rodents (McBee and Bickham 1988), aromatic hydrocarbons with English sole (Jenner et al. 1990), radioisotopes with slider turtles (Lamb et al. 1991), contaminants with great blue herons (Custer et al. 1997), and pesticides with green frogs (Lowcock et al. 1997). Levels of double-stranded DNA breaks were found to be negatively correlated with mosquitofish fecundity (Theodorakis et al. 1997). Studies of this type require specific precautions in sampling and analysis to prevent misinterpretation of tissue-level differences in admixtures of cell types as being indicative of cancerous or other pathological effects (Tiersch and Wachtel 1993). In addition, although the integrity of paternal DNA is crucial for conveying correct genetic material to embryos, fertilization may not be inhibited by certain forms of damage (Ahmadi and Ng 1999).

Several methods exist for analyzing DNA integrity of fresh cells, including comet analysis (Singh et al. 1989, Mitchelmore and Chipman 1998) and the sperm chromatin structure assay (SCSA) (Evenson et al. 1999). Few, if any, methods have yet been designed for direct assessment of DNA fragmentation in fixed sperm cells. However, a flow cytometric method was developed and optimized for testicular cells from yellow perch *Perca flavescens*, largescale suckers (Jenkins, unpublished data), and common carp (Jenkins et al. 2011). Overall, the intranuclear DNA of fixed cells was stained following incubation with acid and heat exposure, making strand breaks more available for intercalation of the DNA stain and consequent detection (Figure 2). The nuclei outside the main population (NOMP) (Jenkins et al. 2011) are analogous to cells outside the main population (COMP) in the SCSA (Evenson et al. 1999).

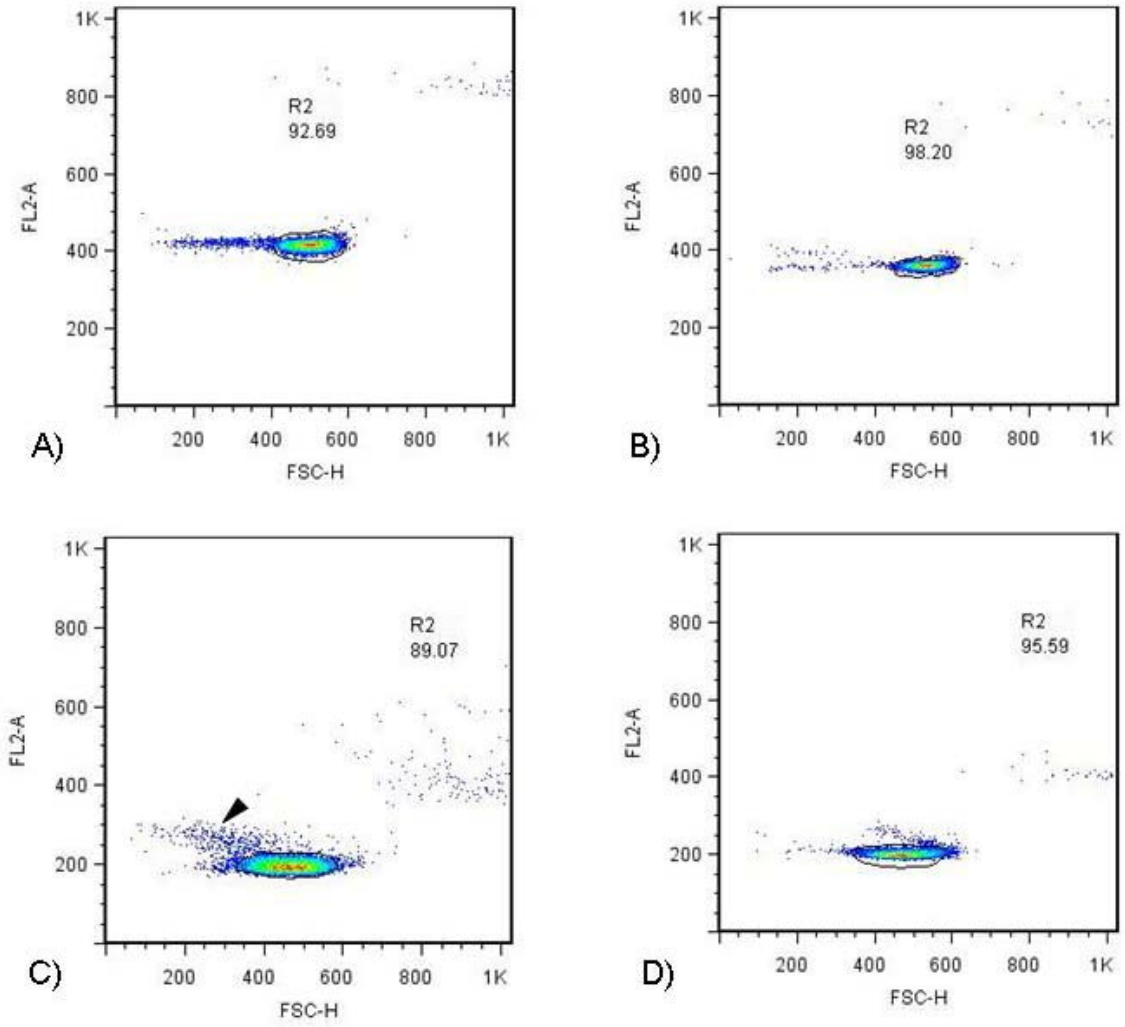

Figure 2. An example of DNA integrity screening using four fish collected from natural water bodies. The top panels are from razorback suckers and show two-dimensional plots of sperm nuclei stained with propidium iodide. In these plots the main population of nuclei appears within a pre-set gate (the oval outlines above, with the corresponding population percentage identified as "R2"). The percentages of nuclei outside this main population (NOMP) (outside the oval gate) were equal to 7.3% (panel A) and 1.8% (B). The NOMP nuclei had fragmented DNA and appeared smaller (indicated by FSC-H) than nuclei within the main population. In one largescale sucker (panel C), 10.9% were NOMP having higher fluorescence (indicated by FL2-A) and smaller size (arrowhead) than those in the main population. Less fragmentation was observed in another largescale sucker (D) with 4.4% NOMP.

Because of the universality of the DNA molecule, agents that are genotoxic for one group of living organisms are typically genotoxic for other groups (Al-Sabti 1985). Thus, biomarkers of molecular genotoxicity can be effective early warning tools. Data on DNA fragmentation levels can be used to screen milt samples for further use, and can be complementary to other sperm quality biomarkers (Jenkins et al. 2011).

Conclusions

Male fish offer procedural advantages compared to females for studying the effects of compounds that cause endocrine disruption. Flow cytometry is a well-established clinical research tool that is well suited for detection of cytotoxic injury in organisms exposed to environmental contaminants (Dallas and Evans 1990). It allows rapid, accurate, and relatively inexpensive cytogenic testing, and because milt, like blood, consists of individual cells in suspension, sperm samples can be easily stained and analyzed for multiple parameters. The gamete quality assays discussed above provide great utility for assessment of reproductive capacity in natural populations, and can be applied in selecting males for artificial spawning, gamete storage, and post-thaw evaluation. As these techniques are developed and further validated, they create opportunities for gamete quality evaluation in aquatic species that could assist widespread adoption and commercialization of germplasm banking by cryopreservation.

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New Chapter

Application of Computer-assisted Sperm Analysis (CASA) to Aquatic Species

Huiping Yang and Terrence R. Tiersch

Introduction

Computer-assisted sperm analysis (CASA) (also referred to as computer-assisted semen analysis) uses computer software to collect, detect, identify, and quantify attributes of motility in a sperm sample. It was first designed for use in humans and livestock, and is considered to be an objective, accurate approach for sperm motility assessment in mammals because it relies on actual counts and measurements rather than subjective observation and estimation. Basically, these systems comprise three components: 1) an optical system; 2) a method for image capture, and 3) data analysis and reporting. Currently, several manufacturers provide complete CASA systems or software (listed in Table 1). Based on the descriptions from the associated user manuals, the basic functions of these systems are similar and are typically based on quality standards developed for human semen put forth by the World Health Organization (World Health Organization 2010).

Table 1. Examples of commercially available systems for computer-assisted sperm analysis.

System name	Manufacturer	Location
Medea LAB	Medea LAB	Bruckberg, Germany
Sperm Vision	Minitube of American	Verona, WI, USA
AndroExpert	AndroExpert	Haus am See, Switzerland
Sperm Quality Analyzer (SQA-V)	Medical Electronic Systems	Los Angeles, CA, USA
Integrated Semen Analysis System (ISAS)	Projects i Serveis R+D S.L.	Valencia, Spain
Sperm Class Analyzer (SCA)	Microptic S. L.	Barcelona, Spain
IVOS sperm analyzer	Hamilton Thorne	Beverly, MA, USA
CEROS sperm analyzer	Hamilton Thorne	Beverly, MA, USA
Image J	National Institutes of Health	Bethesda, MD, USA
Hobson Sperm Tracker	Hobson Vision Ltd	Derbyshire, UK
The CellTrak/S system	Motion Analysis Corporation	Santa Rosa, CA, USA
Sperm Motility Quantifier (SMQ)	Wirson Scientific and Precision Equipment	Auckland Park, South Africa
Olympus Micro Image Analysis	Olympus C&S	Czech Republic
CASAS-QH-Q	Qinghua Tongfang	Beijing, China
Mika motion analyzer software	Medical Technologies Montreux SA	Clarens/Montreux, Switzerland
Image-Pro Plus 5.0	Media Cybernetics, Inc.	Bethesda, MD, USA
Auto sperm*	MedCalc Software bvba	Mariakerke, Belgium

* This device does not require image capture.

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To produce accurate and reliable results by use of CASA, a series of parameters and thresholds in the system need to be properly established to ensure that sperm cells can be recorded and sorted into appropriate categories such as cell size, contrast, and identification of movement. These settings are essential for the application of CASA, and are based on characteristics such as size, shape, and swimming trajectory of sperm from each species. For most CASA systems, these settings can be validated by playing back of videos in sequence and inspecting the frames in real time to confirm if the cells were categorized correctly.

Some Characteristics of Sperm from Aquatic Species

Compared to mammalian sperm, fish sperm possess some specific characteristics and show great diversity among species. Accordingly, the CASA settings for analysis of fish sperm are different from that for mammalian sperm. With respect to size and morphology, fish sperm heads are usually around 2-5 μm, much smaller than those of mammalian sperm (8-10 um). The morphology of fish sperm, especially ultrastructure, varies enough from species to species to be used as phylogenetic criteria (Jamieson 2009).

For motility activation and swimming duration, fish sperm possess characteristics different from mammal sperm. Generally, fish sperm are quiescent in the testes, and their activation relies on the difference in osmotic pressure or ion levels between the testicular fluid and the outside environment (Morisawa and Suzuki 1980, Coward et al. 2002, Alavi and Cosson 2006) and also can be influenced by factors such as pH and temperature (Alavi and Cosson 2005). Upon activation, fish sperm show only a short swimming duration time (from 30 sec to 5 min, except for sperm from live-bearing fishes and some euryhaline fishes), while mammal sperm usually can swim for d. In addition, fish sperm move faster than the mammalian sperm, and the movement trajectory can be different.

As general approaches, collection of fish sperm samples can be performed by stripping or by crushing of testis. These latter samples can include immature sperm cells or somatic cells which require specific thresholding of parameters to distinguish them from sperm cells. Usually the parameters for recording of movement need to be set manually, and in terms of the specific values chosen, can be fairly subjective. This problem is exacerbated in aquatic species because of the great variability in sperm morphology and physiology (Jamieson 2009), and due to the short time of peak motility duration in most species ($\leq$ 30 sec). Therefore, to achieve accurate results in aquatic species, it is necessary to establish suitable parameters concerning image capture, cell size, speed values, light intensity and contrast, and photometer settings for each species based on sperm characteristics and condition (e.g., fresh, refrigerated, or post-thaw samples).

Current Application of CASA in Aquatic Species

Genetic improvement has driven great production gains in livestock industries such as poultry and dairy, and advances have been made for aquatic species (Burnell and Allen 2009). Preservation of valuable germplasm can improve genetic resources and reproduction, and also can be applied to conservation of imperiled species. So far, sperm cryopreservation has been studied in more than 200 species since its beginning in the 1950's (Blaxter 1953), and has been applied to large-bodied aquaculture fish species and small aquarium fishes (e.g., (Yang and Tiersch 2009). However, evaluation of gamete quality is still an extremely important but highly problematic component in sperm cryopreservation. To evaluate male gamete quality, work began

in the late 1970's in mammals to develop objective, automated technologies to rapidly evaluate sperm movement. This led to development of CASA systems that became widely commercially available in the 1990's, and have been adopted in biomedical applications and for use with high-value livestock.

In the late-1980's CASA was first applied to use in fish. Since then (at time of this writing) there have been 62 publications addressing this topic. Of these, 56 are peer-reviewed primary research articles, and 6 are reviews. The bulk of this research addresses demonstration of the feasibility of CASA application in fish (only 2 publications address invertebrates). The types of research address the following topics: sperm characteristics, motility changes after exposure to toxic chemicals and hormone treatments, sperm enzymology, motility characteristics in relation to storage solutions (e. g. pH, buffer, and osmolality), and sperm motility after cryopreservation. Most of the research utilized fresh sperm collected by stripping or crushing of testis, and only 7 of these reports addressed thawed sperm.

Generally, no standardization of methodology exists for CASA application in fish and shellfish. Indeed, 23 of these publications did not include any statements concerning instrument settings (Table 2), and several publications mentioned only certain parameters such as definitions for progressively motile or static cells. Proper parameter settings are essential to ensure that the images collected and analyzed are the targeted sperm cells. Also, 30 of these publications did not report sperm concentration, while 11 provided a dilution ratio only. The type and depth of viewing slides for loading of samples can affect the concentrations determined by CASA, and potentially influence sperm movement. Most publications described the types of slides used, but with large variation in detail. Temperature can be a factor controlling motility, especially swimming velocity. Of the 56 reviewed publications, 26 did not report sample temperature at the time of images capture. In addition, the time interval prior to the start of image capture after motility initiation and the timing of data collection periods are critical factors for analysis of velocity and motility because fish sperm are often motile for only sec to min, and the duration of burst speed can be short (10-20 sec). Rapid sample handing followed by high speed video recording is required to monitor this window. Of the 56 publications, 46 described this in some way, but most lacked information to clarify even if the starting time and period of video capture used for analysis were within the window of maximal sperm motility.

With respect to the output parameters used for sperm quality in these publications, most reported motility, progressive motility, velocity (μm/s) including average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL), and other parameters such as beat cross frequency (BCF), lateral head displacement (ALH), and swimming duration time.

Overall, the previous studies summarized in this review have demonstrated the feasibility of CASA for aquatic species, and showed that several output parameters are useful for evaluating gamete quality. However, routine application of CASA in aquatic species is limited by: 1) lack of clearly established instrument settings, especially for material other than fresh, stripped sperm of fish; 2) lack of standardized protocols, and 3) consequently because of these deficiencies, not taking advantage of the full range of analysis capabilities of these powerful instruments. These shortcomings need to be addressed by systematic evaluation of representative panels of aquatic species from freshwater, marine, euryhaline, anadromous, and catadromous habits with external and internal fertilization.

Table 2. Summary of previous publications addressing the use of CASA for aquatic species (arranged in chronological order).

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Boitano and Omoto 1992	Rainbow trout *Oncorhynchus mykiss*	Fresh	10	--	Regular glass slide	At 10 sec	2 sec	30	Definition of linear, arched, & circular
Toth et al. 1995	Common carp *Cyprinus carpio*	Fresh	23-25	Dilution ratio only	20-um µ-Cell semen chamber (Fertility Technologies)	At 12-14 sec for 1-2 min	15-20 sec; 25-30 sec; 55-60 sec	200	Detailed listing
Christ et al. 1996	Common carp	Fresh	23-25	Hemocy-tometer	20-um µ-Cell semen chamber	--	15-20 sec; 55-60 sec	200	Same as above
Ciereszko et al. 1996	Lake sturgeon *Acipenser fulcescens*	Fresh & thawed	15	Neubauer counting chamber	20- um Microcell (Conception Technologies)	At 5 sec & 5 min for 20-30 sec	25 frames	200	--
Kime et al. 1996	African catfish *Clarias gariepinus*	Fresh	--	Dilution only	--	At 20 sec	15 sec x 4	25	Detailed listing
Ravinder et al. 1997	Common carp	Fresh	2, 3, 25	Dilution only	10-µm Marler chamber (Fertility Technologies)	At 10 sec	--	25 or 60	Detailed listing
Toth et al. 1997	Lake sturgeon	Fresh	12	Dilution only	20-µm µ-cell semen analysis chamber	At 5 sec for 25 sec	--	200	Detailed listing
Creech et al. 1998	Fathead minnow *Pimephelas promelas*	Fresh	RT	60-100 sperm/view	--	At 10 sec x 4	2 sec	30	Cited another reference
Ciereszko et al. 1999	Muskellunge *Esox masquinongy*	Thawed	22	--	20-µm Microcell	At 15-20 sec	25 frames	200	--
Linhart et al. 2000	Common carp	Thawed	--	Dilution only	Regular glass slide	At 10 sec	15 sec	--	Threshold velocity only

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Rurangwa et al. 2001	African catfish	Fresh & thawed	RT	Dilution only	10-well multitest slide with cover slip (ICN Biomedicals)	At 0 sec to 2 min	15 sec at 5 sec after activation	--	Modified settings of Kime et al. 1996
Kime and Tveiten 2002	Spotted wolffish *Anarhichas minor*	Fresh	--	--	12-well slide* with cover slip	At 30 sec	15 sec	--	Detailed listing
Rurangwa et al. 2002	African catfish & common carp	Fresh	--	--	10-well multitest slide with cover slip	At 5 sec to 20 sec	--	--	Kime et al. 1996
Schoenfuss et al. 2002	Goldfish *Carassius auratus*	Fresh	--	--	--	--	--	--	--
Van Look and Kime 2003	Goldfish	Fresh	--	--	12-well slide* with cover slip	At 0 sec	5–20 sec	--	Detailed listing
Elofsson et al. 2003	Fifteen-spined stickleback *Spinachia spinachia*	Fresh	15	--	12-well slide*	At 20 sec	--	--	Detailed listing
Warnecke and Pluta 2003	Common carp	Fresh & thawed	20 ± 1	--	10-µm chamber (Stroemberg/Mika-CMA)	At 15 sec for 5 sec	32 frames	50	Definitions of motility
Aravindaksh an et al. 2004	Spottail shiner *Notropis hudsonius*	Fresh	--	By CASA	--	5 sec	--	--	--
Asturiano et al. 2004	European eel *Anguilla anguilla*	Fresh	--	Hemocy-tometer	Teflon-coated microwells coated with 10% BSA	--	--	--	--

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Burness et al. 2004	Bluegill *Lepomis macrochirus*	Fresh	20	Dilution ratio only	Improved Neubaur hemocytometer	0.5-sec readings x 6 in 90 sec	60 sec after activation	--	Definition of motility
Kleinkauf et al. 2004	Flounder	Fresh	--	--	12-well slide*	At 15 sec	30-45, 45-60, & 60-75 sec	--	--
Le Comber et al. 2004	Three-spined stickleback *Gasterosteus aculeatus*	Fresh	--	Dilution ratio only	12-well slide*	0 to 105 sec at 15-sec intervals	--	--	Detailed listing
Vermeirssen et al. 2004	Atlantic halibut *Hippoglossus hippoglossus*	Fresh	--	--	PTFE-coated slide (ICN Biochemicals)	--	--	--	--
Asturiano et al. 2005	European eel	Fresh	--	Hemocy-tometer	--	--	--	--	--
Burness et al. 2005	Bluegill	Fresh	20 ± 1	Hemocy-tometer	--	10, 20, 30, 45, 60, & 120 sec for 0.5-sec	10 sec after activation	--	Definition for immotile
Dietrich et al. 2005	Rainbow trout	Fresh	20	--	12-well slide* with cover slip	At 5 to 20 sec	--	50	Detailed listing
Urbach et al. 2005	Arctic charr *Salvelinus alpinus*	Fresh	--	--	Micro slide with cover-slip	At 0 sec to 1.5 min	--	50	Contrast,cell size,VAP threshold & VSL
Babiak et al. 2006a	Atlantic halibut	Fresh	6-8	Hemocy-tometer	Counting chamber (Leja products)	At 0 to 105 sec	--	50	Contrast, cell size

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Babiak et al. 2006b	Atlantic halibut	Fresh	20	Dilution ratio only	Burker's chamber	At 30 sec	0.5 sec	--	Defined static cells
Felip et al. 2006	European sea bass	Fresh	--	Dilution ratio only	Regular glass slide	At 5 sec	--	25	Immotile, slow, moderate, & fast
Hu et al. 2006	Amphioxus *Branchiostoma belcheri*	Fresh	RT	Hemocy-tometer	--	At 0.5, 4 & 10 min for 3 sec	--	--	Immotile, swaying, circular & progressive
Kowalski et al. 2006	European smelt *Osmerus eperlanus*	Fresh	4	Dilution ratio only	12-well slide*	At 4 sec for 12 sec	--	--	--
Locatello et al. 2006	Guppy *Poecilia reticulate*	Fresh	26	--	12-µm microcell chamber	--	--	--	Static cells: VAP, VCL & VSL
Holt et al. 2007	Bluegill	Fresh	20	Dilution ratio only	Neubaur hemocytometer	At 0 to 60 sec	5-10 sec	30	--
Liu et al. 2007	Red seabream *Pagrus major*	Fresh & thawed	18-20	--	10-µm chamber (20-ul)	At 10 sec	--	24	Defined motility
Wilson-Leedy and Ingermann 2007	Zebrafish *Danio rerio*	Fresh	20 ± 1	--	12-well (12-µm) slide coated with 1% polyvinyl alcohol with cover slip	At 15 sec	--	97	--
Wojtczak et al. 2007	Common carp	Fresh	--	Spectro-photometer	12-well slide*	At 15 to 30 sec	--	--	--
Cabrita et al. 2008	Senegalese sole *Solea senegalensis*	Fresh	--	--	--	At 15, 30, 45 & 60 sec	--	--	--
Ciereszko et al. 2008	European whitefish *Coregonus lavaretus*	Fresh & thawed	--	--	12-well slide*	At 5 sec	15 sec	--	--

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Dietrich et al. 2008	Rainbow trout	Fresh	RT	Spectro-photometer	12-well slide*with cover slip	At 5 to 20 sec	--	--	Detailed listing
Fitzpatrick et al. 2008	Blue mussel *Mytilus trossulus*	Fresh	--	Yes	1-mm welled slide with cover slip	--	0.33 sec	60	--
Jha et al. 2008	Blue mussel	Fresh	20	--	20-μm chambered slide	--	0.5-sec x 10	60	--
Martinez-Pastor et al. 2008	Senegalese sole	Fresh	--	--	--	At 15, 30, 45 & 60 sec	--	--	--
Singh and Singh 2008	Stinging catfish *Heteropneustes fossilis*	Fresh	--	By CASA	Slide coated with 1% polyvinyl alcohol	At 15 s	--	--	Kime et al. 1996, 2001; Chowdhury and Joy 2001 with modifications
Zilli et al. 2008	Gilthead sea bream *Sparus aurata* & Striped sea bream *Lithognathus mormyrus*	Fresh	--	--	12-well slide* with a cover slip	At 15 sec	45 sec	--	Detailed listing for each species
Gasparini et al. 2009	Guppy	Fresh	--	--	Glass slide coated with silicone with cover slip	--	--	--	Defined static cells
Krol et al. 2009	European smelt *Osmerus eperlanus*	Fresh	--	Yes	Method of Kawalski et al. 2006	--	--	--	--
Ottesen et al. 2009	Atlantic halibut	Fresh	7	--	Standard counting chamber (Leja)	--	--	--	--
Rosengrave et al. 2009a	Chinook salmon *Oncorhynchus tshawytscha*	Fresh	12	--	Regular glass slide with cover slip	At 10 & 20 sec for 0.5 sec	--	--	--

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Rosengrave et al. 2009b	Chinook salmon	Fresh	--	--	--	At 10 & 20 sec for 0.5 sec	--	--	Defined motility
Schoenfuss et al. 2009	Goldfish	Fresh	22	--	--	--	--	--	--
Wilson-Leedy et al. 2009	Zebrafish	Fresh	--	--	Slides coated with 1% polyvinyl alcohol, 0.5-mm perfusion chamber (Invitrogen)	At 0 sec for 150 sec	--	97	Refers to Wilson-Leedy et al. 2007
Zilli et al. 2009	Gilthead sea bream	Fresh	--	--	12-well slide* with cover slip	--	--	--	--
Dietrich et al. 2010	Vendace *Coregonus albula*	Fresh	6	--	12-well slide* with cover slip	--	--	--	--
Groison et al. 2010	European hake *Merluccius merluccius*	Fresh	22	--	--	At 15 sec for 30 or 120 sec	15 sec	25	Detailed listing
Marchand et al. 2010	Mosambique tilapia *Oreochromis mossambicus* & African catfish	Fresh	RT	--	2-ul Leja chamber	At 0 sec for 50 sec	first 10 sec	30	--

--: Not reported.
RT: Room temperature.
*****12-well slides: 12-well multi-test slide from ICN Biomedicals.

Outlook for Future Application of CASA in Aquatic Species

Sperm quality analysis and control are necessary components for a wide range of programs including aquaculture, cryopreservation, and environmental monitoring. Currently, germplasm cryopreservation, distribution, and development represent a multi-billion dollar global industry for improvement in livestock industries. These activities provide a working blueprint for establishing parallel industries in aquatic species, and allow adoption of the equipment originally developed for mammals for use in fish and shellfish such as CASA systems. The publications summarized in Table 2 demonstrated the potential for application of CASA in fish and shellfish. However, to fully integrate CASA into aquaculture or germplasm programs as a reliable tool for evaluation of gamete quality, more investigation is needed. An approach for integration could include the following:

1) As stated above, *standardized settings* are essential for collection of data used for analysis. Data collection by CASA can be entirely dependent on control of settings (such as brightness and contrast) and protocols (such as timing of data capture). Due to the specific characteristics and diversity of fish sperm compared to mammal sperm, a panel of aquatic species to represent freshwater, marine, and euryhaline habitats (including species with distinct motility characteristics such as live-bearers) needs to be evaluated at controlled conditions (e.g., concentration and temperature). Standardized procedures for CASA parameter settings need to be established, and thus can serve as templates for use with new species in the future. In addition, sperm collected by stripping or dissection of the testis (necessary in some species) in fresh, stored, and thawed conditions needs to be compared for parameter settings.

2) *Standardized procedures* for data collection and analysis are needed to ensure reliable results. This is a large problem for several reasons. For example, most fish sperm are motile for 30 sec or less. However, CASA systems are generally designed for use with mammalian sperm which can be continuously motile for d. Thus, rapid data collection is necessary for fish sperm. The interval timing and duration chosen for analysis is critical to ensure observations are made during the time of peak motility. In addition, the problems associated with proper mixing of samples with activating solutions and development of volumetric chambers suitable for use with sperm of aquatic species need to be addressed.

3) Identification of *output parameters* in CASA analysis is most useful for estimation of gamete quality and prediction of sperm viability during refrigerated storage and shipping, after thawing, and in use for fertilization. After locking in the settings and protocols, it will be necessary to link the output parameters available from CASA analysis to sperm fertility for use with aquatic species.

4) Eventual *integration of instrument settings, protocols, and output parameters* into practical methodology would be the goal for application across a broad range of aquatic species. Such an approach would allow specific, systematic and repeatable analysis profiles for sperm before freezing and after thawing, and would allow work to be directly compared across species and laboratories.

The problems encountered in navigating this pathway to standardized CASA application have been addressed previously in livestock species such as cattle, swine, and horses. As such, they could provide useful templates for planning and implementation of informed approaches relevant to aquatic species (e.g., see the Technical Guide for IVOS, TOX IVOS, and CEROS, version 12.3, August 27, 2007, Hamilton Thorne Biosciences, Beverly, MA, USA).

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New Chapter

Computer-aided Semen Analysis

Diarmaid H. Douglas-Hamilton, Kate M. Craig and Tom G. Kenny

"Computer-aided semen analysis", also often referred to as "computer-assisted sperm (or semen) analysis" (abbreviated as CASA in all cases) uses a computer program to detect, identify, and quantify attributes of sperm cells in a sample. In the devices pioneered by Hamilton Thorne, Inc. *(www.hamiltonthorne.com)*, the IVOS model offers a self-contained unit that houses a specialized optical image-forming system combined with the computer. The CEROS model offers an independent computer and microscope. In this chapter we will briefly summarize the features, operation, methods, and limits of CASA instruments using these models as examples.

The Optical System

There can be an external microscope or an internal optical design. The sperm are illuminated and a magnified image is produced by a camera, typically CCD (charge-coupled device), which collects a series of images for analysis. Illumination is often provided by a Kohler condenser, and either a tungsten filament (as in the CEROS model) or a LED (light-emitting diode) source (IVOS). The use of LEDs for illumination has the advantage that they are monochromatic, and can easily be configured as a strobe light, thereby catching details of sperm motion by "freezing" its motion. Either negative phase-contrast or darkfield illumination is best, because detection algorithms can more easily detect bright objects on a dark background than black objects on a grey background (as in positive phase-contrast). While it is possible to detect positive phase-contrast images, the accuracy is lower than using negative phase-contrast. For large sperm such as those of rat we recommend using 4-x darkfield, while for typical farm animal sperm such as pig, bull, or horse we use 10-x or sometimes 20-x negative phase-contrast. A sample containing freely swimming sperm is introduced into the optical system for examination. Capillary-loaded slides (20 μm chambers, e.g., Leja, Amsterdam, Netherlands) are often used as specimen holders, but corrections must be applied to avoid errors in calculating sperm concentration due to Segre-Silberberg loading effects (which cause heterogeneous distribution of the cells within the chamber) (Douglas-Hamilton et al. 2005a,b). Surface-loading (drop-load) slides can also be used.

Image Capture

A series of sequential images is collected of a particular viewing field. Each image is called a frame. Typically, 30 frames may be made at an acquisition rate of 30 Hz or 60 Hz. The sequence of frames is then analyzed.

Setting of Thresholds

Objects are detected by using a contrast threshold and by identifying brighter regions that are connected to each other. The size of the connected region is used as a gate to select only sperm-sized objects. The average pixel intensity of the region can be used as another gate to select only sufficiently bright objects.

Douglas-Hamilton, D. H., K. M. Craig and T. G. Kenny. 2011. Computer-aided Semen Analysis. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 255-260.

Identification of Movement

The sequential frames are examined and their images subtracted. This procedure eliminates static objects and identifies objects that have moved between frames. These objects are generally identified as sperm cells. Their successive position in each frame is noted and stored.

Identification of Sperm

Acquisition of sperm data is enabled by setting the minimum contrast (MC) of the object. This number is the minimum value above background that the pixel brightness must have for that pixel to be potentially part of a sperm image. The minimum size (MS) of the image defines the minimum number of pixels -- all of which must have a brightness value above the MC -- that is required for the object to be potentially identified as a sperm. The MC and MS are set in Analysis Setup and vary depending on the sperm size. For example, large sperm such as porcine have a MS of ~15, while small sperm such as those of zebrafish have a MS of ~3. The choice of MC is typically in the range of 30 – 80 on a scale 0 – 256. It is an inevitable aspect of electronic imaging that as the MC is raised, the object will appear smaller, and *vice versa*. This must be kept in mind while optimizing sperm acquisition accuracy for a given species.

Normalization of Sperm Size

The moving objects are almost all sperm. Occasionally a piece of debris will be "kicked" by the tail of a passing sperm, and may therefore move for several frames, but it will be eliminated from the analysis. We use the moving sperm to normalize size and brightness of the static sperm. We average the size (number of illuminated pixels) and the brightness (integrated intensity of all the pixels in the sperm image), and assume that static sperm have a mean size and brightness that is on average in constant relation to those of the motile sperm. We identify static objects as "sperm" if their size and brightness fall within gating limits in a range around the mean motile size and brightness. These gates can be set by the user, and in general are different for different species. Because static sperm are usually lying on the substrate and swimming sperm are rotating, the apparent intensity and size of a flat-headed sperm (such as bovine or porcine) can be quite different if it is motile or non-motile. Nevertheless in most animals the brightness and size gates work well for identifying static sperm, and because the sperm heads of fish are round (not flattened as in mammals) this problem is reduced for aquatic species. For human samples there is a problem caused by the large amount of debris particles, some of which inevitably fall within the limiting gates. For clean samples as normally encountered in routine practice (e.g., with bull, boar, equine, dolphin, elephant, dog, and most species of fish from which sperm can be stripped) the discrimination works well.

Motility Parameters and Data Reporting

Definitions

The motile objects identified are categorized by quality of motion. Velocity is derived for each sperm, and the velocity is divided into 3 types (Figure 1):

Straight-line velocity (VSL) is the velocity calculated as the object moves in a straight line between the first and last frames.

Curvilinear velocity (VCL) is the velocity taken as the total distance moved between each frame summed over all frames, divided by the time interval between the first and last frames.

Average path velocity (VAP) is made as a running average of the positions making up VCL, and thereby generating a smoothened version of the path.

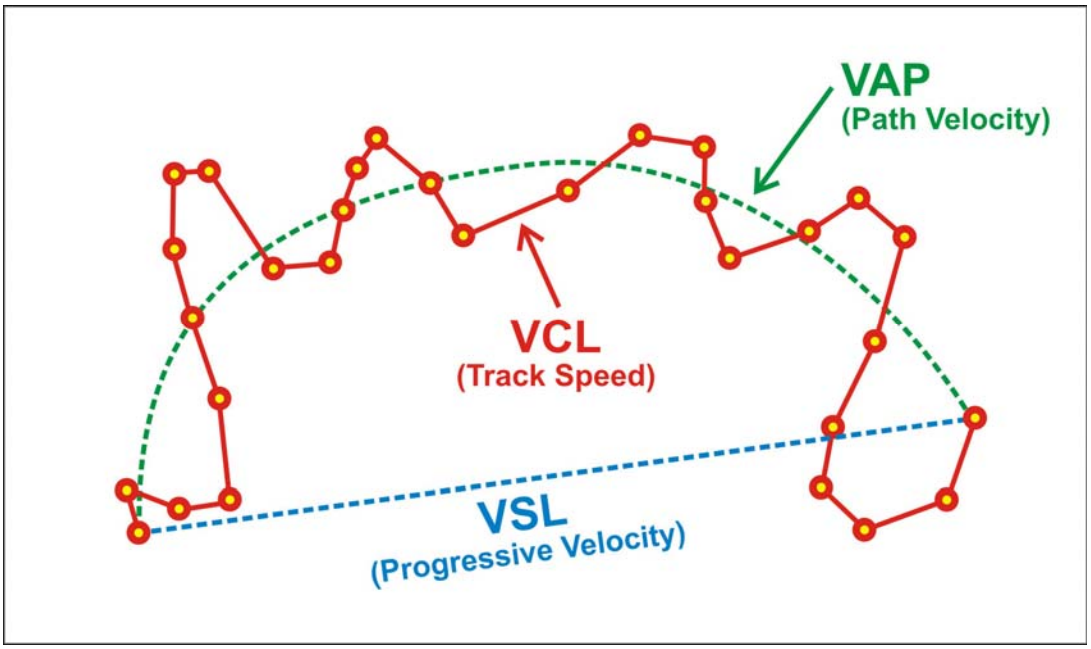

Figure 1. Diagrammatic representation of the track of a sperm as seen by the computer, with VSL, VCL, and VAP indicated.

It is always true that **VCL > VAP > VSL**. The ratios of these velocities indicate how straight the sperm is swimming. We define **linearity (LIN),** as the ratio of **VSL** to **VCL.** We define **straightness** (**STR**) as the ratio of **VSL** to **VAP**. The values of **LIN** and **STR** are always expressed as percentages.

A further useful value is the **motility (MOT**) which is the number of motile sperm divided by the total number of sperm, expressed as a percentage. The **progressive motility** (**PROG**) is more restrictively defined as the number of all motile sperm for which **VAP** and **STR** exceed thresholds set by the user. **Lateral head displacement (ALH)** is the full-wave amplitude of the motion, and **beat-cross frequency (BCF)** is the frequency with which the head crosses the center line, moving in either direction.

Statistical Reporting

The mean value of each of these parameters is reported in the Results screen following analysis, each together with its standard deviation. Bar charts of the distributions are generated. Of course, the distributions of these parameters are not generally Gaussian and the standard deviation is of little value as a statistic, but it is a guide to the breadth of the distribution.

Playback and Validation

It is essential to have a method of verifying whether the analysis has worked properly. This is done by inspecting the series of frames acquired, which are played back in sequence in real time so that the sperm appear to be moving. Superimposed on the image are the tracks of the moving sperm, and the path of the sperm can be inspected to verify that it moves exactly along the computed track. Simultaneously the static cells are identified with red dots. Simple inspection immediately confirms if the static and the motile cells were properly acquired. Iteration between the Playback screen and the Setup Analysis screen to control MC and MS allow rapid feedback to identify the optimum settings.

For accurate work it is essential to verify that the algorithms used by the instrument give true representations of the sperm motion. It is relatively simple to check on the identification of sperm position, by inspection on the Playback screen. Sperm velocity can be confirmed by doing the detailed Pythagorian distance calculations between points, allowing for the image acquisition rate. Computed sperm track, lateral head displacement and beat cross frequency can all be checked directly from the raw data. Methods for this process are summarized in the Instrument Validation Handbook version 12. 3 (Hamilton Thorne, Inc. 2010).

Fluorescent Illumination

The IVOS provides a unique form of fluorescent illumination optimized for examining motile sperm, but also applicable to static cells. Illumination is provided by a xenon flashtube that produces radiation from 330 to 700 nm, which can be used for various dyes after appropriate filtering. The xenon flash lasts on the order of a microsecond, but provides sufficient light during this time to excite fluorescent dyes and allow images to be recorded. One advantage of this metered ultraviolet (UV) exposure is that the illumination is only present during image formation, and consequently phototoxicity is greatly reduced. Another advantage is that moving sperm can be recorded accurately. This is typically done using IDENT, a Hoechst-based dye that stains the DNA in the sperm head and makes sperm easily visible, countable, and measurable even if suspended in obscuring media such as milk or egg yolk. Because this fluorescent xenon illumination can be switched on instantaneously, unlike mercury lamp illuminators, other capabilities such as Viadent (described below) are enabled.

Identification of Sperm Cells in Mixture with Somatic Cells

This combination of UV-xenon illumination and DNA-specific staining that allows precise counts of all cells that contain condensed DNA, such as sperm. Somatic cells can be excluded by brightness because their DNA is less condensed and accordingly less bright. In this way, accurate counts of cells in media containing homogenized somatic cells (e.g. homogenized cauda epidydimis in the case of rat sperm) can be performed (Strader et al. 1996), as well as determination of motility in any type of medium (Tardif et al. 1998). The stain recommended is Hoechst 33342, packaged and sold as **IDENT (**Hamilton Thorne, Beverly, Massachusetts, USA).

Measurement of Membrane Integrity

Another application of fluorescence and the xenon flash tube is to use a dye that penetrates only disrupted membranes, such as Hoechst 33258, marketed in prepackaged form as **VIADENT** (Hamilton Thorne). This dye is added to the medium and rapidly penetrates non-

intact cells, staining the nuclear DNA. Intact cells are penetrated more slowly, allowing one to discriminate intact (viable and non-fluorescent) and non-intact (non-viable and fluorescent) cells. In the VIADENT algorithm, a sample is stained, which does not affect the sperm motility or velocity. The algorithm then automatically does the following:

1. Measures a user-pre-set group of fields and frames in LED visible light, and determine the cell count and motility, as well as progressive motility. This is usually done with a negative phase-contrast objective.
2. The algorithm immediately switches on the xenon illumination and returns to precisely to the first field to repeat viewing the same set of fields in fluorescent light. This will discriminate which of the static cells were viable and which were non-viable. The fluorescent image is normally illuminated and observed through the same negative phase-contrast objective used for the visible image. This objective does not transmit UV illumination as well as a SiO_2 objective, so the images are integrated, typically for 0.25 sec, to provide adequate illumination. These short integration times do not require cooled cameras, and the IVOS captures undistorted, and noise-free integrated images with as long as 3-sec integration times.
3. Superimposition of these images allows visual identification of cells and confirmation of the analysis.
4. The results are combined to generate MOT, PROG, all of the motion parameters, and the fraction of viable cells.

An Alternative Method for Measurement of Membrane Integrity

In a further variant of VIADENT**,** the logic is reversed and the stain H33342, which penetrates membranes and stains all cells, is first applied. This is a useful method of detecting sperm if the medium is obscuring, or if the cells have already been stained, as in samples sorted for X and Y chromosomes by flow cytometry. The cell count, MOT, PROG, and all motion parameters, can be determined on the stained sample under xenon illumination, because all sperm will fluoresce when UV light is applied. Then, a second stain which deactivates H33342 but does not penetrate membranes is applied. When illuminated, the sample will show some of the static sperm as fluorescent and some as non-fluorescent. The non-fluorescent sperm are non-viable, because their membranes have been penetrated by the deactivating dye. The difference between the fluorescent static counts in the two sets of readings provides the non-viable number of sperm.

Operation Logging and Password Protections

These features are available to comply with the requirements of Title 21 of the Code of Federal Regulations, part 11, which addresses electronic records and electronic signatures. These standards are met by the IVOS and CEROS systems.

CASA systems such as the IVOS have been used all over the world for human and animal sperm analysis. Other species that have been analyzed include bull, boar, stallion, zebra, cheetah, leopard, dolphin, turkey, goat, ram, rat, mouse, salmon, trout, and zebrafish. Apart from medical interest for humans, estimation of breeding potential, animal health, and investigation of toxicity effects have been reported.

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New Chapter

Microfluidic Mixers for Standardization of Computer-Assisted Sperm Analysis

Daniel S. Park, Christian Quitadamo, Terrence R. Tiersch and W. Todd Monroe

Overview

Maintenance of rare or valuable genetic material is vital for preservation of biomedical research resources derived from model organisms such as zebrafish *Danio rerio* and medaka *Oryzias latipes*. However, the aquarium fish research community is becoming overwhelmed by the maintenance of thousands of research lines as live populations. Cryopreservation is a proven method for large-scale preservation and maintenance of important genetic material. It extends the reproductive potential of males, reduces the need to maintain live fish, and can prevent catastrophic loss of irreplaceable research lines. Cryopreservation is the most cost-effective alternative for maintaining genetic resources of aquatic organisms, because it can reduce costs for fish and facility maintenance, personnel, and space, and accelerate the development of new research lines.

A cryopreservation program requires post-thaw evaluation of sperm quality on a per sample basis. This challenge is magnified when working with a high-throughput system, when non-wild-type strains are being cryopreserved, and when standardization is required among laboratories. Currently the most commonly used method for estimating quality of fish sperm is evaluation of motility. This has been performed qualitatively by microscopic observation, but now increasingly is performed by use of computer-assisted semen analysis (CASA) systems originally designed for use with human and livestock sperm. Unfortunately, zebrafish sperm can lose peak motility rapidly after activation (within 10 sec) and existing CASA systems cannot begin to capture data rapidly enough to reliably monitor the peak motility phase. This problem is exacerbated by use of volumetric viewing chambers that are slow to fill and are subject to swirling currents that can interfere with the CASA analysis. These challenges have been addressed by performing CASA analysis with open glass slides that allow rapid initiation of data collection, but compromise standardization and eliminates features such as automated cell counting offered by the volumetric chamber. Therefore a need exists to develop microfluidic capabilities to enable rapid mixing of sperm cells with activation solution during transport into a volumetric viewing chamber for reliable and accurate assessment by CASA. In addition, microfluidics offers a new field of opportunity for application with aquatic species gametes.

Sperm Quality Analysis as a Critical Hurdle in Aquatic Germplasm Cryopreservation

In the mid-1990s CASA was first applied to use in aquatic species. Since then there have been 46 publications (including conference abstracts and review articles) addressing this topic (reviewed elsewhere in this volume by Yang and Tiersch). Of these, 26 are peer-reviewed primary research articles. The bulk of this research addresses demonstration of the feasibility of CASA application in fish, and as yet no standardization of methodology exists for aquatic species. This is important because there are a number of fundamental differences between the sperm of mammals and aquatic species including cell size, motility activation mechanisms, and swimming speed and duration. Moreover, very few of these publications address thawed sperm and most utilize fresh sperm collected by stripping. Because this early work was based on

Park, D. S., C. Quitadamo, T. Tiersch and W. T. Monroe. 2011. Microfluidic Mixers for Standardization of Computer-Assisted Sperm Analysis. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 261-272.

demonstrating the feasibility of CASA for aquatic species, it focused on the output parameters and showed that several were useful for evaluating gamete quality. This work shows great promise for CASA use, but routine application is limited by: 1) lack of clearly established instrument settings, especially for material other than fresh, stripped sperm of fish; 2) lack of standardized protocols, and 3) consequently because of these deficiencies, not taking advantage of the full range of analysis capabilities of these powerful instruments. These problems are compounded by a lack of instruments specifically configured for use with aquatic species, especially with respect to the need to rapidly mix sperm and activating solutions and make accurate readings during the peak phase of motility without interference from swirling or other incidental effects that simulate or obscure actual motility.

Microfluidics Technology for Meeting the Challenges of Sperm Quality Analysis

Microfluidics is the field of study involved with the flow and interaction of fluids on a small scale. Generally, the fluids are controlled in environments where the geometrical dimensions are less than 1 mm, power consumption is low, and sample volumes are below the microliter, and are often in the picoliter range. Most microfluidic devices have been utilized in research settings in conjunction with microscopes to visualize flow. Overall device sizes tend to be the size of a standard microscope slide as seen in Figure 1A. In a microfluidic environment, fluids generally move in strictly laminar flow. This is expressed by the dimensionless Reynolds number ($Re = \frac{VL}{v}$, where V is the fluid velocity, L is the characteristic length (or diameter) of the channel, and v is the kinematic viscosity of the fluid). At low Re the flow is classified as laminar, lacking turbulent flow which typically causes mixing of different fluids at a macroscopic scale. Due to the highly laminar flow (Figure 1B) that occurs in small diameter platforms resulting from low Reynolds number (<100), the physics of these platforms are primarily dominated by viscous forces rather than inertial forces. This means the mixing that does occur is strictly from molecular diffusion of the fluids, generally a much slower process than turbulent mixing. However, with the small scale of microfluidics, the gained benefit of low power consumption and the rapid completion of chemical reactions due to high surface-area-to-volume ratio, has gained prominence for the field

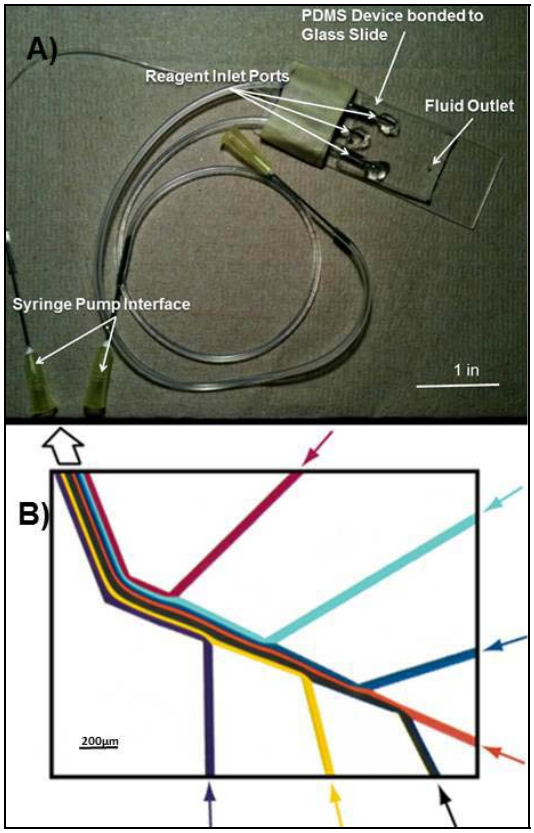

Figure 1. A) Example of a microfabricated chip for sperm analysis, whereby sperm samples are introduced into the microfluidic network through small-bore Tygon tubing. B) Example of laminar flow in a microfluidic network designed by Whitesides and colleagues, where inlet streams of dyed water do not mix. Image in (B) from *Science* cover (2 July 1999) reproduced with permission from AAAS.

in point-of-care diagnostic and care applications as well as processes that require many process steps. These devices, often called Lab-on-a-Chip (LOC) or micro-total-analysis-systems (μTAS) have the ability to integrate multiple laboratory techniques onto a single microfluidic device, potentially reducing the need for large-scale laboratory equipment for many applications such as pathogen detection and gene sequencing.

With respect to more basic and applied aquaculture, microfluidics has yet had little impact. These technologies have been used to shorten analysis times, reduce amount of reagents, and enable new discoveries in cell biology (Beebe et al. 2002), yet few have demonstrated applications for sperm physiology. The exceptions are *in vitro* fertilization studies in mammals, where microchannels have been used for more predictable delivery of sperm to oocytes (Suh et al. 2006), sperm sorting and qualitative selection (Lih et al. 1996, Schuster et al. 2002, Cho et al. 2003, Suh et al. 2005, Seo et al. 2007). A recent report has studied the swimming behavior of bovine sperm in microfluidics, suggesting the influence of side-wall and other microstructures on sperm motility (Lopez-Garcia et al. 2008). While there has been much progress on the mixing of simple solutions in microchannels (e.g., Hessel et al. 2005), no reports exist on the use of microscale fluidic elements to mix solutions containing sperm cells for their activation and assessment.

Design Concepts for Microfluidic Mixers

Microfluidics offers a novel environment to manipulate and study cell activity while reducing costs. With devices the size of a microscope slide, multiple tests can be used to replace larger bench-top analyses. Intricate networks can be created to serve a wide variety of analyses with channels on the order of 100 μm. Because of the absence of turbulence, as stated above, mixing occurs exclusively through molecular diffusion. Therefore, dedicated mixing structures are required to combine sperm samples with activating diluents. Because the physics of flow in these systems is dominated by viscous forces rather than inertial forces, mixing on the microscale has been a notoriously difficult aspect of these platforms, so a component in most microfluidic devices is the micromixer. There are numerous designs that attempt to mix fluids efficiently, and all micromixers are characterized as either active or passive (reviewed in Nguyen and Wu 2005). Active micromixers involve the recruitment of an external field or force to increase perturbation in fluids present in the device. These micromixers are typically more difficult to fabricate and as a result are more costly and less often achieve implementation beyond laboratory experimentation. Passive micromixers utilize specialized channel geometries to increase mixing efficiency by increasing the interface area between fluids with differing concentrations of solutes and solvents. This increases chaotic advection of fluid particles and promotes diffusion of solutions down their concentration gradients. While passive micromixers are typically the most accessible forms of mixers due to their comparable simplicity to active mixers, they tend to be less effective at fully mixing the desired reagents.

A compromise must be determined based on the desired application of mixing. For instance, when mixing aquatic sperm, “complete” mixing may not be required so long as the concentration passes the needed threshold for the desired reaction. Similarly, activated sperm may enhance mixing by the beating of their flagella. It is important to note that traditional active mixers such as electro-osmotic mixers may interfere with the normal activity of the characterized cells leading to artificial results that become difficult to interpret. Passive mixers could also interfere with normal cell activity by introducing a greater amount of shear stress through

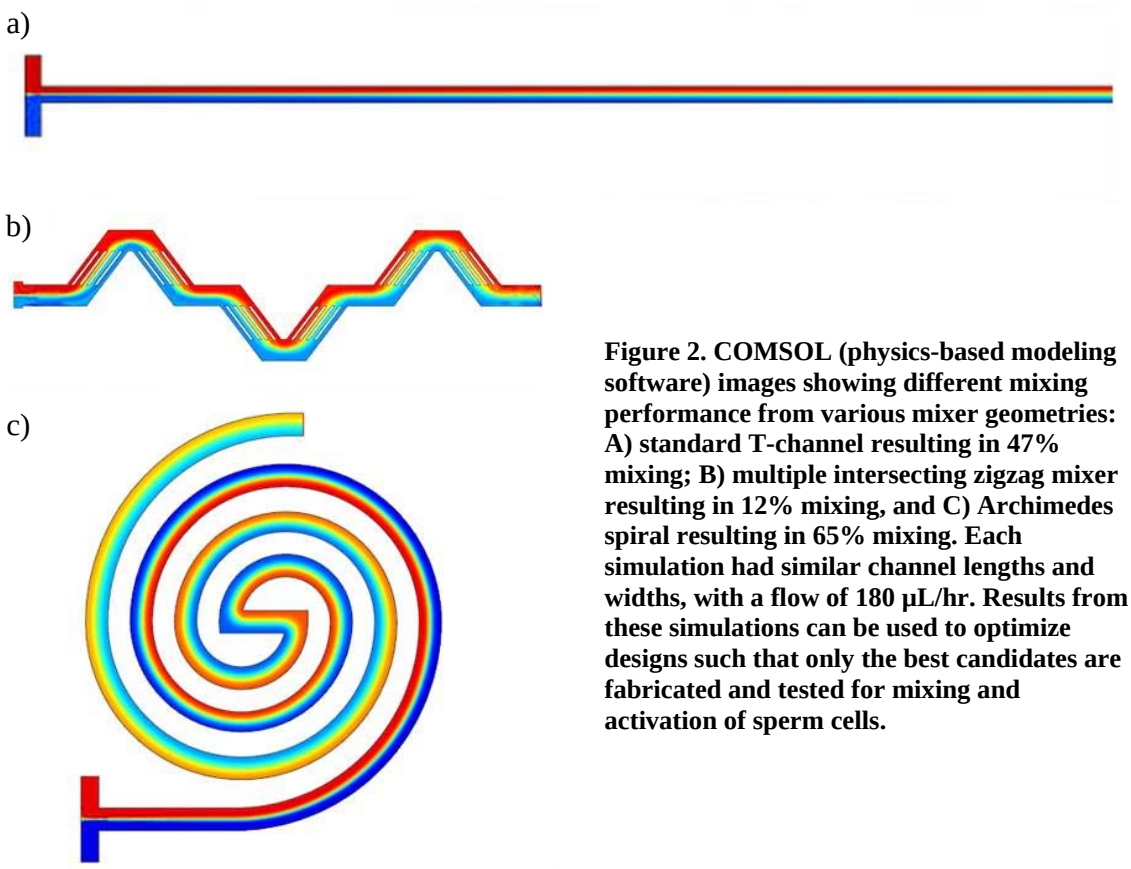

Figure 2. COMSOL (physics-based modeling software) images showing different mixing performance from various mixer geometries: A) standard T-channel resulting in 47% mixing; B) multiple intersecting zigzag mixer resulting in 12% mixing, and C) Archimedes spiral resulting in 65% mixing. Each simulation had similar channel lengths and widths, with a flow of 180 µL/hr. Results from these simulations can be used to optimize designs such that only the best candidates are fabricated and tested for mixing and activation of sperm cells.

complex channel geometries and increased fluid velocities. Constraints such as these require design of passive mixers with simple geometries and high mixing efficiency with a short residence time in the device.

As stated above, because the microfluidic environment is so different than the macrofluidic environments that we are typically exposed to, design of such a mixer can be difficult and expensive in time and materials. At the beginning of design, the required geometries for rapid, effective, non-destructive mixing are often unknown, and this typically requires much prototyping and preliminary testing. However, computing technology has now advanced enough to allow a broader population of researchers to use multiphysics modeling software packages (such as COMSOL, *www.comsol.com*) to predict various properties of microfluidic devices. A micromixer geometry for example can be drawn in a computer-aided design (CAD) software and imported into a modeling application where the fluidic properties (such as density, viscosity, and diffusion coefficient) of the expected reagents can be inputted along with various flow parameters. The final results from the modeling yield a fairly accurate simulation of the micromixer with information such as flow velocity, Reynolds number, and most importantly, concentration at any given point (Figure 2). A workflow of design and modeling can be utilized to design and redesign the micromixers to determine the optimal geometry for the micromixer before any prototype is fabricated. After several candidates have been shown to have effective mixing results in the simulations, these mixers can be fabricated and tested with dyes to evaluate mixing in a real world setting. The use of this modeling technique will not always yield perfect results, however; utilizing it can guide the development process to save resources and optimize the design of the microfluidic structures.

Microfabrication Methods of Microfluidic Devices for Sperm Analysis

The overall fabrication process of microfluidic devices consists of multiple steps including design, microfabrication, generation of through-holes, sealing, and incorporation of macro-to-micro interfaces (Figure 3A). Design of microfluidic devices based on performance simulation results is carried out using CAD tools prior to microfabrication processes. Generation of microstructures for microfluidic devices is preceded either by fabrication of a mask or fabrication of a master mold (Figure 3B). Masks are fabricated by either patterning of a thin metal layer on glass, or high-resolution printing on a transparency (Weibel et al. 2007). Fabrication of master molds are done typically by lithography of photoresist (Whitesides et al. 2001), high-precision micro-milling of metal such as brass (Hupert et al. 2007), or electroplating of nickel (Park et al. 2008).

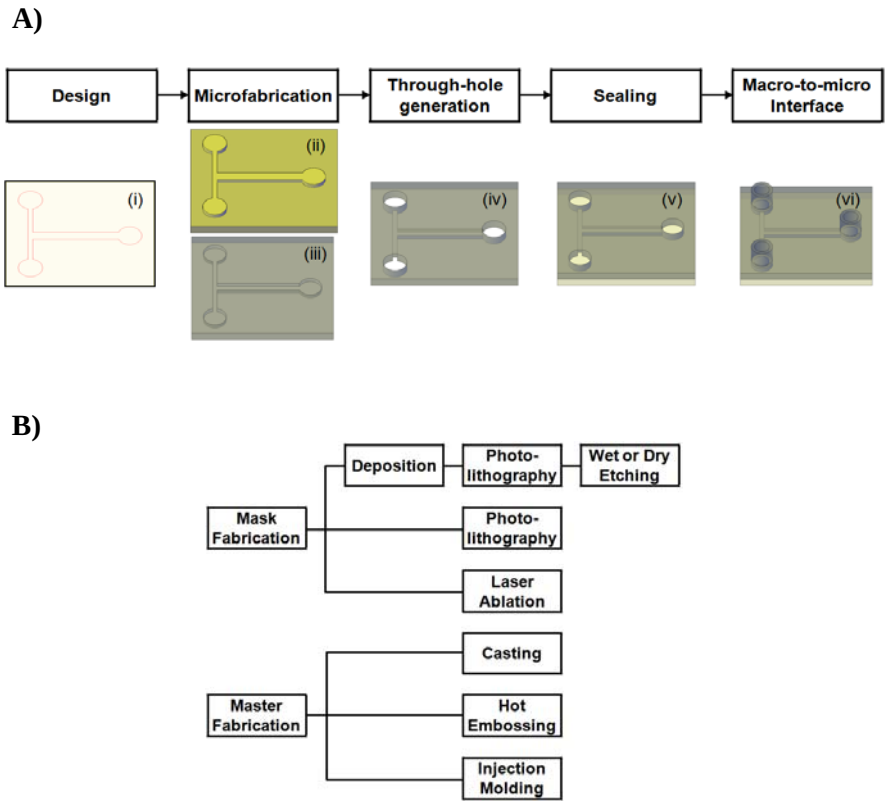

Figure 3. A) The overall fabrication process of microfluidic devices: i) design of a microfluidic device, ii) fabrication of a master mold, iii) fabrication of a microfluidic device, iv) generation of through-holes, v) sealing, and vi) incorporation of macro-to-micro interface. B) The steps in microfabrication processes commonly used for fabrication of microfluidic devices.

Methods of generating features and devices on scales from nanometers to micrometers have been put forth by the well-established semiconductor industry for purposes of creating integrated circuits, where smaller circuit features equates to faster microchips. These same techniques have enabled the fabrication of microfluidic devices on silicon or glass by use of deposition of metal or dielectric materials, lithography of photoresist materials through a

fabricated mask, and wet or dry etching (Beebe et al. 2002). Wet etching of silicon is commonly carried out with potassium hydroxide for anisotropic etching (Zou et al. 2005) and wet etching of glass with hydrofluoric acid for isotropic etching (Liu et al.2006). Dry etching of silicon is preferred for fabrication of high aspect ratio structures using tetrafluoromethane gas or sulfur hexafluoride gas chemistries (Nagrath et al. 2007). Those approaches have been applied to the study of gametes, such as manipulation of single oocytes in silicon microfluidic devices using wet and dry etching (Zeggari et al. 2007). With glass devices using wet etching, studies have been carried out for separation of mammalian sperm from epithelial cells (Horsman et al. 2005), on-chip cell lysis and DNA extraction from sperm cells (Bienvenue et al. 2006), and on-chip determination of spermatozoa concentration (Segerink et al. 2010). Glass was one of the first materials of choice because it allows for constant observation of on-chip processes via standard light microscopy. From a fabrication perspective, glass was chosen because of its well-established fabrication techniques, but it also carries the drawbacks of high material and process costs, and limited disposability for microfluidic devices (Fiorini and Chiu 2005). Other microfabrication methods are available for fabrication of microfluidic devices including direct patterning of thermoset polymers by photolithography (Sikanen et al. 2010) and laser ablation (Malek 2006a, Malek 2006b).

Microfabrication of polymer devices with fabricated master molds in the past decade has taken advantage of their low cost and disposability. Soft lithography of polydimethylsiloxane (PDMS) casting against a photoresist master mold has become a typical method due to its low material costs, simple microfabrication process, and disposability. Examples include human applications such as: *in vitro* fertilization (Suh et al. 2006), gamete and embryo isolation and culture (Suh et al. 2003, Smith et al. 2007), sperm sorting and separation (Cho et al. 2003, Suh et al. 2005, Wu et al. 2006, Seo et al. 2007), and study of swimming behavior with bovine sperm (Lopez-Garcia et al. 2008). Micro-molding of thermoplastics such as polymethyl methacrylate (PMMA), polycarbonate (PC), and cycloolefin copolymers (COC) can be utilized via hot embossing or injection molding (Becker and Gärtner 2008) to overcome the limited compatibility of PDMS to organic solvents and low microfabrication throughput and to open new opportunities for study of sperm physiology. Table 1 shows the summary of microfabrication methods and materials for microfluidic devices, providing general guidelines for choice of methods and materials of microfluidic devices for sperm analysis.

Table 1. Summary of microfabrication methods and materials for microfluidic devices.

Materials	Silicon	Glass	PDMS	Thermoplastics	
Microstructure generation	Wet or dry etching	Wet etching	Casting	Hot embossing	Injection molding
Material cost	Medium	Medium-high	Low	Low	Low
Process difficulty	Medium	Medium	Easy	Easy-medium	Medium
Process cost	High	Medium-high	Low	Medium	Medium-high
Fabrication throughput	Low	Low	Low	Medium	High
Solvent resistance	High	High	Low	Low-medium	Low-medium

Post-microfabrication processes including generating through-holes, sealing, and incorporating macro-to-micro interfaces are used to complete functional microfluidic devices. Through-holes for fluidic access are commonly generated by drilling or punching of holes to which macro tubing is connected (Liu et al. 2006, Christensen et al. 2005). Sealing of

microfluidic devices is carried out by a variety of methods such as anodic bonding, thermal fusion bonding, adhesive bonding, solvent-assisted bonding, welding, surface modification, and lamination (Zhang et al. 2006, Tsao et al. 2009). For fluidic handling, a connection between a microfluidic device and the macro-environment such as a syringe pump is required to minimize pressure drop, dead volume, and leakage. This present challenges in utilizing microfluidic devices in real world settings and are often referred to as “macro-to-micro interfaces”, interconnects, or “world-to-chip interface” (Fredrickson and Fan 2004). Common methods of macro-to-micro interface are direct tubing with epoxy (Park et al. 2010), PDMS interconnects (Li and Chen, 2003), pressure-fit of a reusable needle with a luer connector (Christensen et al. 2005), integrated interface using self-aligned interconnects with flanged tubing (Puntambekar and Ahn 2002), and embedded interconnecting ports in polymer chips (Lee and Kwon 2010). Modular interfaces with a housing including capillaries, tubes, or other macrofluidic components as a separate module can be used for high-throughput analysis (Nittis et al. 2001, Yang and Maeda 2003).

Preliminary Studies of Microfluidic Devices for Fish Sperm Activation and Analysis

During the spring of 2009 we worked with an undergraduate design team to create a micromixer capable of activating zebrafish sperm. The goal of the project was to create a microfluidic device that could mix a sperm sample with water for activation and analysis via CASA (Figure 4). Our initial design criteria were to evaluate a variety of micro-architectural designs and geometries for mixing capability, and to deliver a 1 µl sperm sample to a defined volume for analysis by CASA within 15 sec of activation. We evaluated two mixing designs, a herringbone depression chaotic mixer (Stroock et al. 2002) and a combination of multiple intersecting and a zigzag microchannel design (Bing et al. 2001, Mengeaud et al. 2002). Molds of microfluidic channels with both features were prototyped using a micromill. A positive brass master was hot-embossed into a PMMA blank which was used as a negative mold. Advantages of this approach were the reduction in machining marks at the surface of channels and chambers, which can occlude transparency. A feature resolution of 0.1 µm, precision of ±1 µm, and aspect ratio of 10:1 (depth:width) was produced with this system, and the mold was filled with PDMS which, following curing, was bonded to a glass slide. Both designs contained two inlets so that dye could be injected through one and water through the other to evaluate mixing based on the design features. Syringe pumps (NE-500, New Era Pump Systems, Inc.) were used to drive matched flows between the two inlets at rates from 50 to 250 µl/hr.

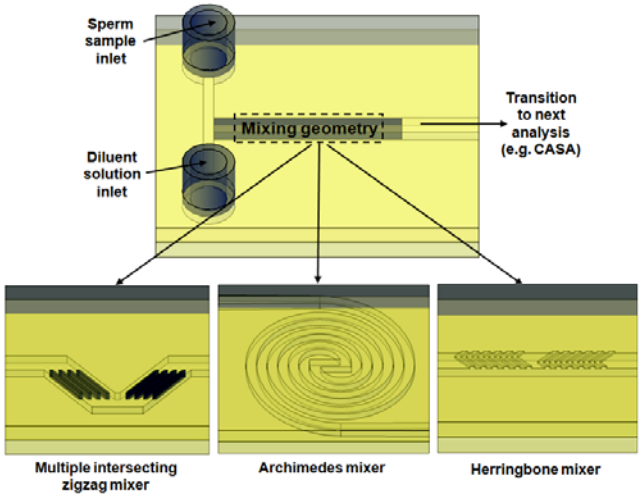

Figure 4. Schematic diagrams of a microfluidic device and three candidate geometries of micromixers (multiple intersecting zigzag, Archimedes, and herringbone) for mixing of sperm with a diluent solution for activation and delivery to CASA.

Light microscopy was used to visualize mixing (Figure 5) where the herringbone features showed superior performance over the multiple intersecting zigzag microchannel design. Inspection of the inlets of both designs showed the challenge of mixing in these microchannels where flow is essentially laminar (with Reynold's numbers of <10). A boundary of sharp contrast can be seen between the neighboring dye and water flow streams, with no mixing until subjected to mixing geometries.

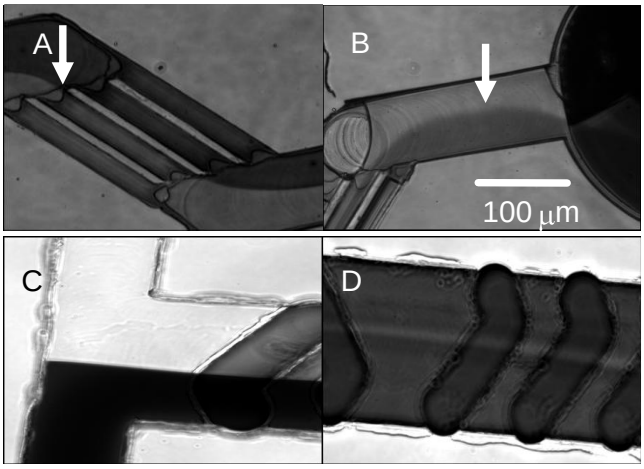

Figure 5. Multiple intersecting zigzag microchannels (A-B) and herringbone mixers (C-D) for mixing in a microfluidic channel. The herringbone design provided more complete mixing by the end of the microchannel (D) than did the multiple intersecting zigzag design (arrow indicates unmixed fluid in B).

Based on the preliminary success of the herringbone mixer adapted for use at these scales, we performed a more quantitative analysis of mixing using fluorescein dye (1 mM) in one inlet which enabled more sensitive probing of mixing over several series of mixing elements in a microchannel (Figure 6). To quantify mixing as a function of length along the channel (or residence time at a given flowrate,) *Equation 1* (Johnson et al. 2002) was used, where N represents the number of measurements taken for a distance, I_i represents the intensity recorded for event *i*, I_i^0 represents the intensity with no fluorescence, and $I_i^{perf\ mix}$ represents the intensity of a perfectly mixed solution. The equation relates the difference of a recorded intensity to a perfectly mixed solution and one with no fluorescence. The results of this design indicate that 95% mixing efficiency can be achieved in less than 2 sec with 100 µl/hr flow rates in the herringbone mixer (Figure 7).

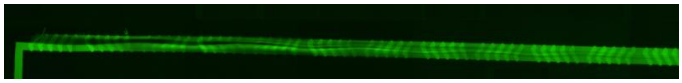

Figure 6. Collage of fluorescent images showing water (top left inlet) mixing with fluorescein (bottom left inlet) while flowing (100 µl/hr) along a series of herringbone elements.

$$Eqn\ 1:\ \text{Mixing Efficiency} = \left(1 - \frac{\sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(I_i - I_i^{perf.mix}\right)^2}}{\sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(I_i^0 - I_i^{perf.mix}\right)^2}}\right) \times 100$$

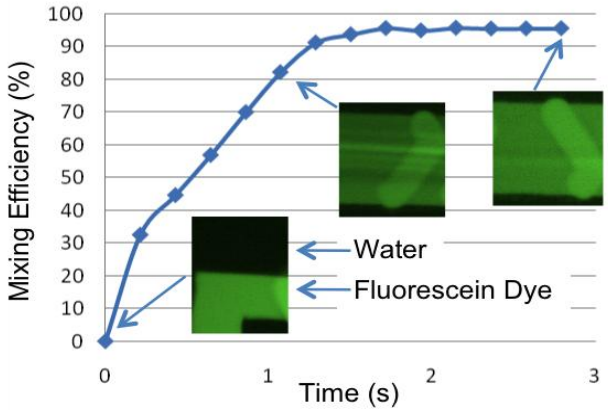

Figure 7. Mixing of fluorescein and water across herringbone mixing elements occurred in less than 2 sec (at 100 µl/hr) in the prototype design.

We evaluated the ability of the herringbone device to activate zebrafish sperm by mixing with water to induce activation. In this case, the PDMS mixing device was bonded to a standard CASA slide and evaluated on a CASA system

(CEROS model, Hamilton-Thorne). Sperm samples mixed with isotonic HBSS showed no motility, negating shear or other device effects on activating sperm. Sperm samples from the same stock were activated by hand (48% motile) and in the device (52% motile) to evaluate if zebrafish sperm could be activated by the device and analyzed by CASA. It was also noted that delivery of the sample into the chamber was accomplished within 10 sec after cessation of flow, with minimal drift within the chamber. The lack of drift achieved with this system may overcome the significant problem of swirling currents that occur often in hand-mixing and complicate accurate CASA measurements. These preliminary results show the promise of microfluidics for sperm analysis. Further design optimization is necessary to obtain effective mixing elements that protect sperm from damage. Expansion of microfluidic devices to include new functionalities beyond mixing and CASA will be necessary to accomplish more robust processing and testing of sperm physiology.

Future Application of Microfluidics to Assessment of Sperm Motility

Subjective estimation of sperm motility is the most frequently used measure of sperm quality in aquatic species (Terner 1986, Tiersch and Mazik 2000). Motility estimates are most reliably performed using darkfield microscopy. The use of CASA instruments specifically configured for use with aquatic species will significantly advance study and banking of aquatic germplasm. Acitivities such as the routine assessment of sperm quality for fresh and post-thaw samples, and determination of sperm activation curves as a function of osmolality will be accelerated by the availability of microfluidic devices, where successive solutions can be rapidly mixed, analyzed by CASA, and rinsed through the device to increase throughput. Similarly, the effects of cryoprotectants and other potential toxins on sperm viability could be studied in shorter time frames following exposure compared to the time scales of current protocols. It is hoped that the application these types of design principles to marine sperm analysis will also enable higher throughput analysis in order to meet the demands of the increasing numbers of model organisms requiring cryopreservation. It is important to note that work of this type is only possible through interdisciplinary collaboration combining engineering, microfabrication, and biology.

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III. Basics of Cryopreservation

Principles of Embryo Cryopreservation

Richard S. Denniston, Sherri Michelet, Kenneth R. Bondioli and Robert A. Godke

Introduction

Gamete and embryo cryopreservation have played a key role in the development of assisted reproductive technologies for farm animals over the last several decades. Embryo transfer was first explored by young Walter Heape, then a student in Cambridge England, in the late 1800s (Heape 1891). Little did young Walter Heape know that his success transferring microscopic rabbit embryos and the production of live offspring would subsequently lead to the development of embryo cryopreservation and transfer procedures for a multitude of mammalian species.

Polge et al. (1949) discovered that the addition of glycerol to the freezing medium could serve as an effective cryoprotectant for animal sperm. Using the freezing method of Polge and co-workers (1949), Stewart (1951) reported the first frozen-thawed sperm calf using glycerol as the cryoprotectant. Five cows were artificially inseminated, resulting in the first mammal (a calf named "Frosty') born from frozen-thawed semen in the world in England.

Smith (1953) reported that low temperatures were not necessarily lethal to animal cells as originally had been proposed but that further research was needed to develop successful gamete and embryo freezing protocols for mammals. Whittingham et al. (1972) and Wilmut (1972) first reported that mouse embryos could be successfully frozen at -196 ^{o}C and then producing live offspring, and that the use of slow cooling and thawing rates were essential to embryo survival. These findings were followed by the first live calf born in 1973 from the transfer of a thawed bovine embryo by one of these two research groups (Wilmut and Rowson 1973). Shortly thereafter, the first frozen-thawed embryo lambs was produced in England in 1976 (Willadsen et al. 1976) and the first frozen-thawed goat offspring were reported in Australia in 1976 (Bilton and Moore 1976). Subsequently, the first frozen-thawed embryo foal produced in Japan in 1982 (Yamamoto et al. 1982) and then followed by a subsequent frozen-thawed embryo foal reported from Colorado State University in 1984 (Takeda et al. 1984).

Since then, the practical storage of embryos from domestic species at freezing temperatures then became a common topic in the scientific literature. Research on the freezing of bovine embryos has generally been based on the approach previously established for freezing of mouse embryos. This was because mouse embryos gave early indications that they were more freeze tolerant than those of other species and thus, became the animal model for developing methods of cryopreserving embryos of domestic animal species.

Wilmut et al. (1975) initially reported that the stage of embryonic development played an important role in survival rates of thawed bovine embryos. A series of experiments provided evidence that cooling late morula and blastocyst stage embryos at 7 or 8 d after fertilization resulted in greater survival rates when compared with early-stage morulae. It was also evident that different species exhibited variability in optimal embryo stage for freezing, as evidenced by mouse embryos, that survive freezing and thawing at much earlier stages of embryo development than do embryos of larger animals (Leibo 1985). Subsequently, Willadsen (1977) concluded that embryos at the late morula to early blastocyst stage of development could be successfully slow-cooled to -36 °C prior to plunging into liquid nitrogen (LN_2). Until the development of this

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protocol, slow-freezing intervals took greater than 7 hr (as temperatures declined to -60 °C or less) prior to plunging the embryos into LN_2.

Embryo freezing protocols became more consistent in response to improvements in freezing equipment and physical alterations in the procedures. The use of 0.25-mL French plastic semen straws in freezing bovine embryos when compared with the traditional use of glass ampules resulted in similar pregnancy rates after embryo transfer. Although post-thaw survival rates were initially slightly higher using the ampules for freezing, easier seeding during the process fostered the development of the plastic straw method of freezing (Massip et al. 1979), which provided uniformity of sperm freezing. Freezing embryos in 0.25-mL straws then became the standard used in the animal embryo transfer industry in the early 1980s (Wright 1985), where it still remains the method of choice today.

Cryopreservation research conducted during the mid 1970s has provided an adequate foundation for the development of the basic methodology for freezing of most mammalian embryos. The standard protocol for mice had been established by the late 1970s, allowing the focus to be on perfecting the freezing methodology cattle embryos. During that era and continuing through to the present, a multitude of freezing protocols have been developed for various laboratory and domestic animal embryos. These efforts have successfully resulted in live viable offspring from at least 14 mammalian species (Table 1).

Table 1. Examples of offspring produced from mammalian embryo cryopreservation (modified from Rall 1993).

Species	Reference
Mouse	Whittingham et al. 1972
Cow	Wilmut and Rowson 1973
Rabbit	Bank and Maurer 1974
Rat	Whittingham 1975
Sheep	Willadsen et al. 1976
Goat	Bilton and Moore 1976
Horse	Yamamoto et al. 1982
Man	Downy et al. 1985
Eland	Kramer et al. 1983
Baboon	Pope et al. 1984
Cynomolgus monkey	Balmaceda et al. 1986
Marmoset monkey	Summers et al. 1987
Cat	Dresser et al. 1988
Pig	Hayashi et al. 1989

Clearly, advances have been made in the past few decades relating to embryo freezing technology. Most of the research efforts have been conducted on cattle, goat and sheep embryos, with more recent efforts focusing on horse and swine embryos. In addition, this technology also allows for the transport and storage of valuable genetic material between progressive seedstock producers (Fahning and Garcia 1992), and serves as a conservation tool in the building of gamete and embryo cryobanks for rare domestic and minor animal breeds, as well as, for endangered animal species.

Principles of Cryopreservation

Cell Preservation

Most of the chemical events associated with freezing are a result of the osmotic properties of cells. The cell is made up of water, organelles, dissolved salts, sugars, proteins and lipids, all surrounded by a semi-permeable membrane. This membrane permits water to flow relatively freely across it, while the membrane acts as a barrier to the larger molecular solutes. By nature, the water tends to move across the cell membrane to maintain an equal concentration of solutes inside and outside the cells. The concentration of solutes in a solution is measured as osmolality, and is typically in the range of 280 to 310 mOsmol for most cell culture media. This osmolality range is approximately the same as the concentration of solutes found inside most living animal cells.

Intracellular and extracellular environments associated with water transport and membrane permeability form the basis for the cryopreservation of cells. In general, larger cells that are less permeable and should be frozen slowly, and those that are smaller and more permeable should be frozen more rapidly (Mazur 1965). The reason behind this concept deals with cellular damage during the freezing process by osmotic shock, intracellular ice formation, increased intracellular concentration of solutes and solution effects (changes resulting from dehydration of cells) (Mazur 1977).

When cells are placed in a medium of equal osmolality (i.e., an equal concentration of solutes inside and outside the cells) they become isotonic. If cells are placed in a hypertonic medium containing a higher concentration of solutes than are inside the cells, water will move out of the cells until an equilibrium is reached. When cells are placed in a hypotonic solution containing a lower concentration of solutes than inside the cells, water will move into the cells until an equilibrium is reached. This results because the solutes inside the cells have relatively large molecular weights and generally do not passively cross the cell membrane. Thus, it is water movement across cell membrane (osmosis) that is responsible for maintaining osmotic equilibrium.

Osmotic properties of mammalian cells attempt to maintain an equilibrium between the concentration of intracellular and extracellular water and solutes. The osmolality of a solution is a measurement of solute concentration and is a critical factor to the success of the cell freezing process. This measurement defines whether a cell or a group of cells (e.g., embryo) is in a hypertonic, hypotonic or isotonic environment. Hypertonic solutions cause water to be released from the cell, hypotonic solutions cause an uptake of water into the cell and isotonic solutions maintain the equilibrium. These actions are crucial to the movement of the cryoprotectant solutions during the freezing procedure. The addition of cryoprotectants usually interact with the cell membranes to make them more ‘flexible’ and thus, reduces damage due to solution effects (Mazur 1977).

Ice Formation in Solutions

The freezing point of pure water is at 0 ^{o}C. Water containing ions and other materials freezes at lower temperatures depending on the solute concentration. In a standard solution, as pure water freezes out of suspension leaving behind a solution of increased solute concentration. The freezing point of the remaining solution will therefore decline. If the temperature is further decreased more water is removed in the form of ice and the concentration of solutes in the remaining liquid increases, which results in a continuous drop in the freezing point of the

solution. As long as the ambient temperature continues to decline, these changes occur until the entire solvent and its contained solutes (and other substances) freeze completely at the eutectic point.

Supercooling and Seeding

A second concept involved during the cryopreservation is that of temperature changes and its effect on cells. For long term cold storage, cells need to be frozen at gradually decreasing temperatures due to the fact that the freezing point of a solution decreases as the level of solute concentration increases. This is the case during the cell freezing process, water freezes out of a solution forming ice crystals, which can disrupt cell membranes. The freezing point continually decreases until the eutectic point is reached and the entire solution solidifies.

The area of concern in freezing solutions is that of 'supercooling.' This is defined as the ability of a liquid to cool several degrees below its own freezing point, yet still remains in the liquid state. This is an unstable state that often results in spontaneous freezing. The irregular structure of the resulting ice crystals can then puncture the cell membrane destroying the cell (Mazur 1963, Leibo 1980). This fluxing activity may be controlled to some extent by altering the cooling procedure.

The process of spontaneous freezing is often referred to as 'nucleation.' This occurs when a supercooled solution warms up to its freezing point, as a result of the heat released when liquid changes into a solid state. To help prevent the formation of these deadly ice crystals, cells need be dehydrated as much as possible; however, the dehydration process only begins following nucleation. Seeding the freezing solution (inducing ice formation in a controlled area of the cell sample) when it is just below its normal freezing point can induce ice crystallization. To do this with the solution holding the embryos, a pair of metal forceps are cooled in LN_2 (-196 ^{o}C) and applied to the plastic freezing straw at a point of contact above the embryo so as to reduce embryo damage in the freezing process.

During cooling, the temperature of a solution can fall below the true freezing point as the solution supercools. This supercooled solution then transforms into a unique metastable state. Depending on the volume of liquid and solute concentration, at an appropriate temperature spontaneous nucleation occurs causing ice crystals to form. At this point, the heat of fusion that is created from changing the liquid to a solid is released. This release of energy causes the supercooled solution to warm to its actual freezing point.

Dehydration may also be aided by decreasing the eutectic point of the cells in solution by adding a cryoprotectant with a lower eutectic point. As the temperature continues to decrease during the freezing process, water is removed from the cells leading to dehydration and less ice formation, once the eutectic point is reached (Rall et al. 1983, Fahy 1986). Rapid freezing may lead to the formation of lethal-size ice crystals, but reduces solution effects. Slow freezing increases the chance of damage due to solution effects, but usually prevents the formation of large irregular ice crystals.

The dehydration of cells does not begin until nucleation occurs. Therefore, when a liquid sample is allowed to supercool to a temperature far below its freezing point, the cells in that sample have not had the same time interval to dehydrate as cells that are in a sample that does not supercool. Large degrees of supercooling may be avoided by artificially seeding the sample at a temperature slightly below its freezing point. Nucleation of the liquid in a metastable state tends to be a random event, and may even be caused by vibration of the cell sample. In early studies, the introduction of a small ice crystal into the liquid was the original method used to induce nucleation.

A more practical method, such as touching the supercooled liquid container with a cold stainless steel instrument has become the method of choice.

Embryo freezing techniques employ four basic steps that have been explored in various domestic and nondomestic species. These steps include the addition of a cell cryoprotectant, the freezing process (which involves cooling, seeding, freezing and storage in LN_2), the thawing process and then removal of the cryoprotectant from the cells of the sample.

Cryoprotectants

Cryoprotectants must possess properties of low toxicity to cells and high water solubility. These cryoprotectants can be classified as either penetrative or nonpenetrative, depending on their ability to pass through the cell membrane. Embryo donor species, temperature, available cell surface area, developmental stage and concentration gradients between the intracellular and extracellular fluids all affect the degree of cryoprotectant permeability for different cell types (Leibo et al. 1970, Mazur et al. 1974, Schneider and Mazur 1984). A list of the most common cryoprotectants used for mammalian embryos is presented in Table 2.

Table 2. The type of permeability of common cryoprotective agents.

Cryoprotectant	Type of action
Dimethyl sulfoxide (DMSO)	Penetrative
Glycerol	Penetrative
Ethylene glycol	Penetrative
Methanol	Penetrative
Dimethyl acetamide	Penetrative
Polyvinyl pyrrolidone (PVP)	Nonpenetrative
Hydroxyethyl starch (HES)	Nonpenetrative
Dextrans	Nonpenetrative
Albumin	Nonpenetrative
Polyethylene glycol	Nonpenetrative

Cryoprotective agents are usually classified as two general types based on their ability to permeate cell membranes. Low molecular weight molecules, such as glycerol, dimethyl sulfoxide (DMSO), propylene glycol and ethylene glycol are classified as intracellular cryoprotectants since they pass through the cell membrane with relative ease. Larger molecular weight molecules, such as sucrose (and other carbohydrates) and protein molecules are termed extracellular cryoprotectants because they generally do not pass through the cell membrane during a short exposure interval.

The most commonly used cryoprotectants for farm animal embryos are DMSO, glycerol and more recently ethylene glycol. Various species exhibit different membrane permeability at different temperatures and stages of embryo development with each of these chemical agents. Although glycerol was one of the first known cryoprotectants to be used in gamete and embryo cryopreservation, research has shown that both DMSO and ethylene glycol are slightly more permeable to animal embryos (Whittingham 1980). Wilmut (1972) evaluated the use of polyvinyl pyrrolidone (PVP) as a cryoprotectant in animal embryos but concluded this agent was not very efficacious. Other cryoprotective agents, with lower cell toxicity properties, have been tested using the various embryo models (Fahy et al. 1984), and are in use today.

The addition of cryoprotectants to cells in solution causes the release of water from the cells as the concentration equilibrium is maintained. This cell dehydration can be lethal if it

occurs too rapidly, therefore, cryoprotectant solutions should be added to the medium in a series of increasing concentrations (Leibo et al. 1974). It has been well established that most of the cryoprotectants penetrate embryonic membranes at room temperature or slightly higher, thus allowing equilibration of the solute concentrations (Fahning and Garcia 1992).

The beneficial effects provided by cryoprotectants during the freezing process is said to be an accidental discovery in 1948 by Dr. Smith and Dr. Polge in Cambridge, England. These researchers had been unsuccessful in attempts at using levulose solutions to freeze fowl spermatozoa and decided to put the project on the shelf for a time. Months later when they resumed their studies, they found one of the 'freezing' solutions that they retrieved from cold storage was successful at preserving motility of fowl sperm cooled to -79 °C. Further evaluation indicated that this solution also preserved fertilizing capability of the sperm after thawing. These encouraging results led the researchers to examine more closely the 'mysterious' solution they had used in their experiment. Chemical analysis showed it contained water, glycerol and animal proteins. Apparently, the glycerol and albumin solution of the resident laboratory histologist had become mixed up with the levulose solutions the researchers were initially testing. After this discovery, the research of cryobiologists quickly focused on the cell protective effects of glycerol and proteins.

The exact mechanism of action of cryoprotectants on embryonic and somatic cells during cryopreservation still remains unclear. It is clear, however, that cryoprotectants are essential for the preservation of animal cells at low temperatures. Because effective cryoprotectants lower the freezing point of whatever solution they are added to, less ice crystals will form at any given temperature. Consequently, the solute concentration in the residual cryoprotectant solution at a given temperature will also be reduced. The detrimental effects of high solute content on cells also give all indications of being temperature dependent. In a cryoprotective solution, the temperature at which cells will be exposed to a high solute concentration will be reduced and thus, will be less damaging to the cells. Most cryoprotectants act on the lipids of the cell membranes during the freezing process to make them more pliable and less likely to be damaged during the process. It is well known that cryoprotective agents reduce the freezing damage to cells by affecting the size and often the shape of ice crystals that form during the freezing process.

Movement of Intracellular Cryoprotectants Across the Cell Membrane

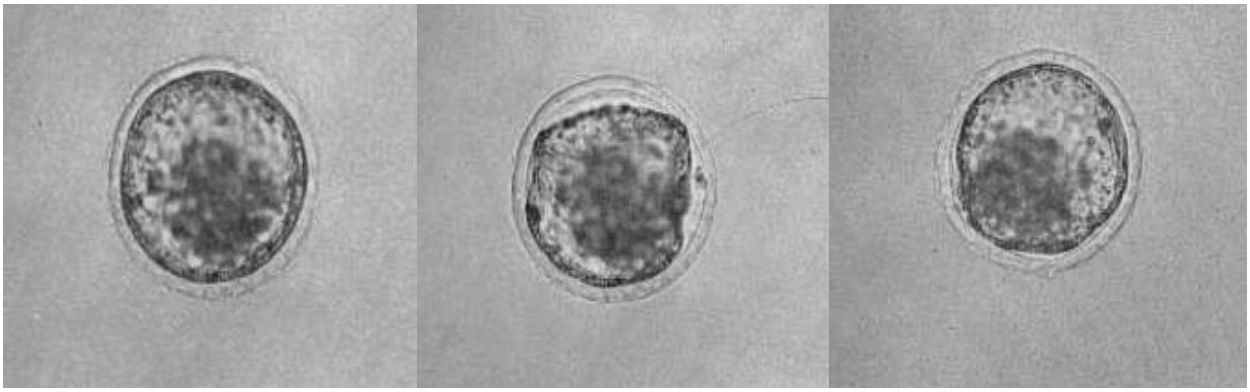
Small molecular weight cryoprotective agents can easily permeate most animal cells. However, water is an even smaller molecule and thus, will more rapidly pass across cell membranes. When cells are placed into a cryoprotective solution, they respond by shrinking as water quickly leaves the cell to dilute the cryoprotectant on the outside of the cells. Within a short period of time, cryoprotectant enters the cells so that the intracellular concentration is the same as the extracellular concentration. Under these circumstances, water re-enters the cells so that cell size is approximately the same as before the cryoprotectant was added to the medium (Figure 1). The marked shrinking and re-expansion of viable cells exposed to a potent intracellular cryoprotectant is often detrimental to the embryo. An alternative is to expose the cells to a series of cryoprotectant solutions with increasing concentrations of the cryoprotective agent. This series of solutions minimizes the degree of cell shrinking, however, the cells still experience contractions and re-expansions, but these fluctuations are not as detrimental to the embryo.

A more serious problem occurs when a cryoprotectant is removed from the embryo. When cells containing a cryoprotectant are placed in cryoprotectant-free medium, water rapidly moves into the cells to dilute the cryoprotectant faster than the cryoprotectant can move out of the cells. This causes the cells to swell, and often burst causing irreversible damage. To alleviate this

problem, cells are placed in a series of solutions with increasing concentration of the cryoprotectant. The cells are allowed to equilibrate in each solution before being moved to the next solution. In this way, the cells swell only slightly before reaching an equilibrium before being transferred to the next solution. It should not be overlooked that many cryoprotectants can be toxic to cells; therefore, the time the embryo is exposed to each solution should be kept to a minimum.

An alternative approach is to use an extracellular cryoprotectant to equilibrate the cells. With this procedure, cells are placed in a freezing solution containing an equimolar concentration of a non-penetrating cryoprotectant (Table 2). In this situation, the tendency for water to move into the cells is offset by the tendency for water to move out of the cells to dilute the extracellular cryoprotectant. The cells will not swell; they initially shrink as the cryoprotectant and the water leave the cells. These cells will return to their normal volume as equilibrium is reached.

Another approach to altering intracellular cryoprotectant in embryonic cells is to use a combination of the previous two steps. Viable cells are placed in a series of solutions of increasing concentrations of intracellular cryoprotectants prior to the freezing procedure and then decreasing concentrations of extracellular cryoprotectants after thawing. In each case, a short equilibration time is allowed for each step. With this methodology, there is generally reduced swelling or shrinking of the embryonic cells.



Bovine expanded blastocyst in PBS holding medium

Bovine expanded blastocyst in PBS holding medium with 5% glycerol (1 min)

Bovine expanded blastocyst in PBS holding medium with 5% glycerol (8 min)

Figure 1. A bovine blastocyst in PBS holding medium with and without glycerol.

Methodical removal of cryoprotectants is also critical in the survival of cells. A risk of cell rupture can occur when thawed cells containing cryoprotectants are placed directly in holding medium lacking cryoprotectant. If this occurs, water rushes into the cell to balance the concentrations of intracellular components with those of the existing extracellular components. Often the larger molecular weight molecules do not exit the cell as rapidly, causing cell damage

(Leibo and Mazur 1978). In contrast, addition and removal of cryprotectants should be completed in a graded series of concentrations. It has been reported that use of a single-step addition of cryoprotectant can produce adequate thawing success in various animal species, although this approach is not used extensively in farm animals (Neimann 1991).

Caution should always be taken when adding cryoprotectants in step-wise or single-step fashion, because these agents are often quite toxic to embryonic cells just prior to hatching. Other options include the use of non-permeating cryoprotectants, such as sucrose. These agents increase the concentration of extracellular solute causing water to exit the cell, which can help balance sudden rehydration after thawing (Leibo and Mazur 1978). At present, some of the most popular methods combine the advantages of both procedures using step-wise dilutions coupled with permeating and non-permeating agents. Early studies with cattle have shown higher survival rates when thawing embryos diluted in a glycerol and sucrose in the step-wise combination method (Niemann et al. 1982).

Cooling Rates

The rate at which cells are cooled during the freezing process is also crucial to post-thaw survival. As previously mentioned, if embryos are cooled too rapidly lethal ice crystals often form. Conversely, if the embryos are cooled too slowly, an increased exposure to solution effects can result (Rall and Polge 1984). This is due to the fact that membrane permeability is related to temperature and exposure time as well as to cryoprotectant concentration and time to equilibration. Slow cooling rates generally range from -0.1 to -0.5 ^{o}C per min when embryos enter a supercooled state (Rall et al. 1983). As the cooling process continues, ice crystals form at between -10 ^{o} and -20 ^{o}C (Mazur 1965). To delay ice crystal formation and allow gradual equilibration, cryoprotectants are used to decrease spontaneous crystal development to -38 to -44 ^{o}C (Leibo 1977, Rall et al. 1983). After the nucleation process has occurred, the cell is no longer permeable to the cryoprotectant and extracellular solute concentration increases. Then the concentration equilibrium can be maintained to -25 ^{o}C if a rate of equal to or less than -1 ^{o}C per min is used in the during cooling process (Mazur et al. 1984).

Cooling rates are of key importance to successful cell cryopreservation. If cells are cooled too rapidly, adequate dehydration does not occur and intracellular ice formation will cause damage to cell organelles and membrane layers. If the cooling rate is too slow, cells will be exposed to a high concentration of solutes for an extended time period. This condition is known as the “solution effect” and is damaging to the cells. Thus, cell damage can occur from too slow or too rapid cooling procedures, although there are two different mechanisms involved in inducing this damage.

Cooling rates can be influenced by the speed with which water moves across a cell membrane. The ability of water to pass across a viable cell membrane is determined by four factors: specific type of cell membrane, temperature, membrane surface to volume ratio and solute concentration. Each cell type has specific membrane characteristics relating to their permeability of water, and these characteristics are generally not easily altered. All other things being equal, water will move slower across cell membranes at lower temperatures. More time is required for the same amount of water to pass through a cell membrane at lower temperatures than at high temperatures. In addition, water will move in and out of smaller cells (with a higher membrane to volume ratio) faster than with larger cells. For this reason, it is usually necessary to cool large cells slower than small cells. Finally, water will move faster to dilute a very concentrated solution compared with a less concentrated solution, with the rate of movement being proportional to the difference in concentration of solutes on each side of the cell membrane.

Warming Rates

Optimal warming rates are dependent on cooling rates and the temperature at the time of plunging the sample in LN_2. When a frozen cell sample is removed from the LN_2 and warmed, the extracellular ice begins to melt and water rapidly moves into the cells to dilute the high concentration of intracellular solutes. The rehydration of the cells is a function of the thawing rate, and is completed shortly after the sample has totally thawed.

When viable cells are cooled at relatively slow rates (0.1 ^{o}C per min to 0.5 ^{o}C per min) and plunged into LN_2 at relatively high temperatures (-30 ^{o}C to -40 ^{o}C), there is usually only a small amount of water remaining in the cells. This water freezes as soon as the sample is plunged into LN_2 forming small-sized intracellular ice crystals. These smaller-sized ice crystals reduce the chance of cell organelle and membrane damage. If this sample is warmed rapidly (>1000 ^{o}C per min), rehydration occurs quickly increasing cell survival rates. However, if this sample is warmed slowly, the intracellular ice crystals can grow larger and recrystallize during the process, increasing the chance of damaging the cell membrane and organelles.

Cell survival during and after thawing is also dependent upon the rate at which the frozen sample is warmed. The warming rate selected is dependent upon cooling rate and temperature prior to plunging the sample into LN_2. Early embryo freezing procedures used slow thawing protocols, warming at rates of less than 25 ^{o}C per min (Whittingham et al. 1972, Rall 1981). With this approach, extracellular ice crystals will melt causing an influx of water back into the cell. If cells are cooled slowly, then smaller ice crystals form inside of the cell. If the sample is warmed slowly, there is a risk of reforming larger crystals that will damage the cell. Rapid thawing of the sample, however, will allow for rapid rehydration and markedly reduce ice damage. This method warms thawed embryos at rates of between 250 ^{o}C and 500 °C per min (Rall and Polge 1984).

Classical Approaches to Freezing Mammalian Embryos

There are a multitude of variables that must be considered when selecting a method for embryo cryopreservation. Protocols differ among species regarding such factors as choice of cryoprotectants (Table 3), cooling rate and stage of embryonic development. Various methods have been described and evaluated for a number of common animal species, including the mouse, rat, rabbit, cat, cow, sheep, goat, horse, pig and primates.

Slow Cooling Procedure

Once donor embryos are harvested, they are placed in sterile holding medium for morphological evaluation. It is recommended that only good quality embryos be selected for freezing. With this procedure, glycerol is commonly used as a 5% or 10% solution. The step-wise equilibration method calls for the embryo to be placed in a 5% glycerol solution for 5 min before transfer to a 10% glycerol solution for 5 min prior to cooling.

Table 3. Percentages of bovine embryos surviving and developing to expanded or hatched blastocysts after freezing in different cryoprotectants (from Takagi et al. 1994).

Cryoprotectant	Embryo survival	Expanded blastocysts	Hatching blastocysts
Glycerol	55%	41%	54%
Propylene glycol	54%	43%	50%
Ethylene glycol (EG)	63%	48%	46%
EG monomethyl ether	56%	43%	49%

After the cryoprotectant has reached an equilibrium, the embryo is loaded into a 0.25-mL plastic freezing straw and placed into an automated freezing machine and is cooled to -6 °C. Once the straw is cooled, it is seeded and held at this temperature for ~10 min. The straws are then inspected to make sure the seeded ice crystals are still present. Care should be taken not to allow temperature fluctuations during this process. Each manual step should be performed as rapidly as possible. After verification of the seeding process, the straw is cooled at a rate of -0.3 °C per min until it reaches -33 °C. At this point, the frozen straw containing the embryo is plunged into LN_2. At this stage, the cooling rate is ~700 °C per min. All indications at the present time are that animal embryos prepared and processed in this manner can remain viable almost indefinitely (Leibo 1985).

The standard way to thaw an animal embryo is to remove the plastic straw from the LN_2 and to place it horizontally across a Petri dish to allow air warming. After 2 min the heat-sealed end of the straw is removed and the embryo is emptied into a sterile Petri dish. Another thawing option is to remove the straw from the LN_2 and submerge it in a 37 ^{o}C water bath. Although this approach is faster, it may result in more embryo cell damage.

At present, the step-wise cryoprotectant removal method is still considered to be the safest method for thawing of bovine embryos. After the embryo is removed from the straw it is placed in a solution of 6.6% glycerol and 0.3 M sucrose for 5 min, transferred to a 3.3% glycerol and 0.3 M sucrose solution for 5 min, then to a 0% glycerol and 0.3 M sucrose solution for 5 min and finally to modified phosphate-buffered saline (PBS). The embryos are allowed to equilibrate in the PBS solution and are transferred individually to recipient females.

This slow cooling approach is similar to the popular slow cooling method that is now used frequently to freeze d-7 cattle embryos (Van Wagtendonk-De Leeuw et al. 1995). Pregnancy rates using conventional methods for good quality (grades 1 and 2) embryos have been reported to be between 50 and 60% (Niemann 1991), and today as high as 75% with good quality recipient females. The step-wise removal of the cryoprotectants is the most frequently used approach, although this method is more time consuming than with other methods.

More Recent Freezing Methodologies

Sperm Stored in the Epididymus

Viable sperm have resulted after freezing and then thawing whole dog postmortem testicles in a household freezer at Louisiana State University in 1997 (Graff et al. 1997). Also, viable sperm have been harvested from the epididymis of postmortem testes of dogs stored for at 4ºC for 24 hr (Stilley et al. 2000).

Epididymal sperm from a male goat has been successfully frozen, thawed, and produced

live offspring (Blash et al. 2000). Although numerous attempts have been made with cattle over the yr, the first epididymal sperm IVF embryo pregnancies were produced at Louisiana State University (Godke et al. 2004). In addition, frozen-thawed cattle epididymal sperm has produced live calves following intracytoplasmic sperm injection (ICSI) (Guerrero et al. 2008) and artificial insemination (Guerrero et al. 2009) at this institution. The use of epididymal sperm for IVF and/or ICSI is especially important when a genetically valuable breeding male accidentally dies or is injured and no longer able to mate naturally. More recently, the first frozen-thawed sperm offspring in mammals (mice) have been born after freezing the male's whole body after death. The males were frozen for 15 yr prior to the harvesting of sperm (Ogonuki et al. 2007). These interesting findings will clearly lead to other studies on epididymal sperm of domestic and nondomestic animals in the future.

Direct Embryo Transfer

Leibo (1984) developed a direct embryo transfer technique for cattle where the frozen embryo is thawed while inside the plastic straw and immediately transferred to the recipient female. This method allows the glycerol to be removed from the embryo while inside in the plastic straw. A sucrose solution is placed in part of the same straw separated from the embryo-containing solution by an air bubble. After thawing, the straw is shaken forcing the embryo-containing solution through the air bubble into the sucrose, which rehydrates the embryo. The direct transfer method has been used in field trials with d-7 cattle embryos and provided acceptable pregnancy rates (Chupin et al. 1984, Leibo 1984, Voelkel and Hu 1992).

This basic one-step freezing procedure for cattle has been modified since it was first developed. An example of a modified one-step method was used by Iwasaki et al. (1994) in assessing embryo freezing effects on embryo inner cell mass (ICM) survival in cattle. With this approach, blastocysts were placed in a 1.36 M glycerol solution (in PBS with 20% calf serum) at 15 °C for 10 min. The embryos were loaded into the straws and were separated from 0.25 M sucrose diluent by two air bubbles. After cooling (at 1°C per min to -6 °C) and seeding, the straws were held for 10 min, cooled (at 0.3 °C per min to -35 °C) and plunged into LN_2. The straws were air-thawed for 10 sec and placed in a 36 °C water bath. The straws were removed from the water bath and inverted, forcing the embryos into the sucrose diluent. To test the system before in-field use, the embryos were washed in PBS with 20% calf serum and co-cultured on cumulus cells until hatching from their zonae pellucidae. This approach was also found to be successful.

Vitrification

Vitrification was first used as a cryopreservation technique with mouse embryos (Rall and Fahy 1985), and has recently has become a viable alternative to the traditional freezing protocols for prehatched embryos. Success has been reported with vitrification of embryos in cow (Massip et al. 1986), rat (Kono et al. 1988), rabbit (Smorag et al. 1989), goat (Yuswiati and Holtz 1990), sheep (Schiewe et al. 1991), the domestic cat (Rall 1993) and more recently in the horse. Unlike conventional embryo freezing media, the vitrification solutions are cooled at very rapid rates (1,000 to 2,000 °C per min). Vitrification is the process of forming a glass-like solid without the formation of ice crystals. These cooled solutions allow for direct plunging of embryos into LN_2. This process may offer some practical benefits in cryopreservation, including saving embryo handling time and eliminating the need for an expensive programmable freezing machine.

Vitrification typically uses a combination of permeating and non-permeating cryoprotectants (Table 4). The cryoprotectants are used at such a high concentration that they dehydrate the cells prior to the initiation of the cooling process. Vitrification solutions share three common properties. First, these vitrification solutions contain a combination of low and high molecular weight cryoprotectants. Low molecular weight cryoprotectants penetrate cell membranes and protect the cytoplasm from damage during cooling. Higher molecular weight cryoprotectants do not pass across the cell membrane, however, they are effective extracellular dehydration agents. Secondly, the final overall concentration of the cryoprotective agents in the mixture is higher than usual, to enhance vitrification and thus, avoiding lethal ice crystal formation. Finally, the standard vitrification solution contains an isotonic level of saline.

During a typical vitrification procedure, viable embryos are equilibrated in a 1.5 M concentration of cryoprotectants. As in the standard controlled-rate freezing protocol, embryos will shrink as water leaves the cells in response to the increased concentration of the solute. Following a short equilibration period, the cells then return to their initial volume. The embryo suspension is then transferred to the final concentration of cryoprotectants in a one-step or two-step procedure prior to plunging of the embryo and its vitrification mixture into LN_2. This short exposure to high concentrations of cryoprotectant agents causes rapid cellular dehydration, preventing intracellular ice formation in the embryo during the cooling process.

Table 4. Examples of commonly used vitrification solutions used in embryo cryopreservation (from Rall 1993).

	Cryoprotective agents[a]		
Solution	**Permeating (M)**	**Non-permeating (%W/V)**	**Reference**
VS1	DMSO (2.62 M) Acetamide (2.62 M) Propylene glycol (1.3 M)	Polyethylene glycol (6%)	Rall 1987
VS2	Propylene glycol (5.5 M)	Polyethylene glycol or BSA (6%)	Rall 1987
VS3	Glycerol (6.5 M)	Polyethylene glycol or BSA (6%)	Rall 1987
Massip's VS	Glycerol (2.2 M) Propylene glycol (3.2 M)	BSA (0.4%)	Massip et al. 1986
Kassai's EFS	Ethylene glycol (7.2 M)	Ficoll (18%) Sucrose (0.3 M)	Kasai et al. 1990

[a]DMSO = dimethyl sulfoxide, BSA = bovine serum albumin, Ficoll = polymer of sucrose.

Pioneering studies on mouse embryos vitrified at freezing rates of ~3,000 ^{o}C per min have resulted in high percentages of post-thaw viability (Rall and Fahy 1985). The rule of thumb established by Mazur (1977) has long been, the faster the embryos are frozen, the smaller the ice crystals tend to be, and hence less structural damage to the cells. These rapid cooling rates decrease embryo toxicity due to an inverse relationship between cooling rate and embryo exposure to high concentrations of cryprotectant. Being able to plunge cells in vitrification solutions into LN_2 is an efficient and economical method to rapidly cryopreserve mammalian embryos (Ali and Shelton 1993).

Oocyte Freezing

Frozen-thawed human oocytes were first used to produce the IVF embryo pregnancies in the mid to late1980s (Chen 1986, Van Uem et al. 1987). This methodology remained dormant for a number of yr, however, the use of this technology has increased in recent yr. First foals from oocyte vitrification were produced at Colorado State University in 2002 (Maclellan et al. 2002). This procedure along with oocyte transfer is becoming the procedure of choice for 'problem breeder' mares. During the last 8 yr, vitrified cattle oocytes have produced viable IVF and nuclear transfer embryos, and offspring have been born. This area in farm animals is under intense investigation at the present time.

Refreezing Embryos

Refrozen intact embryos (mice) have resulted in viable post-thaw embryos at Louisiana State University in1992 (Vitale et al. 1992). Also, successful refreezing of mouse embryos after blastomere removal was subsequently reported in 1993 (Snabes et al. 1993). The first report of refrozen cattle embryos producing viable post-thaw embryos was reported at Louisiana State University in 1994 (Vitale et al.1994). The ability to successfully refreeze embryos offers additional opportunities in developing new assisted reproductive technologies in the future.

Summary

The first procedures used during farm animal embryo cryopreservation, in many cases, were damaging to the embryo and thus, during the first yr, births of live, viable offspring (e.g., cattle) were only sparingly reported. Advances in the understanding of the mechanisms causing freezing injury led to the substitution of some of the agents initially used, such as the cryoprotectant, DMSO. This agent was subsequently found to be quite toxic to embryos, causing researchers to continue seeking alternatives. Developing new procedures and fine tuning existing freezing protocols was followed by a progressive increase in the post-thaw embryo pregnancy rates during in-field trials in a multitude of livestock herds. Today, new freezing and warming protocols are constantly being developed and tested, and research is directed towards obtaining practical and reliable methods for gamete and embryo cryopreservation, like that of direct embryo transfer that is now used in commercial cattle operations.

Embryo cryopreservation procedures in farm animals have produced a number of effective protocols now used in other species. Acceptable pregnancy rates for frozen-thawed embryos have been demonstrated in the cow, goat, sheep and more recently with early-stage horse embryos. Over the yr, the pig has required more research attention to produce the first frozen embryo offspring than did other farm animals. At this stage, commercial embryo freezing and thawing protocols are not yet available for the commercial swine producers.

At present, various cryoprotectants, rates of cooling, and procedures for thawing and cryoprotectant removal have been explored in farm animal species. However, the economic benefits of cryopreservation and cryobanking will continue to drive the research efforts. Even with the important advances made to date, further research is needed to develop novel methods, so that pregnancy rates of thawed embryos will be similar to those of fresh transferred embryos. For example, with *in vivo*-produced swine embryos and *in vitro*-produced ovine, caprine, porcine, bovine and equine embryos, we are still far from reaching that level.

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Original Chapter: Revised by Authors

Cryopreservation of Bull Sperm

John E. Chandler and Robert A. Godke

Background

The use of artificial insemination (AI) in cattle, specifically dairy cattle, grew out of the desire to use the best genetically available sires to enhance milk production. This concept was originally started by what was known as "bull clubs." The local clubs were composed of interested farmers who realized the benefit of using the sire as a means to achieve genetic improvement (Figure 1). The sire was important because one bull can produce multiple offspring per yr, whereas, a cow can only produce 1 or perhaps 2 calves, in the case of twins (occurs at a low frequency in cattle) per yr. Thus, the main reason for the success of artificial insemination is the desire for genetic improvement. The first commercial cattle artificial breeding service cooperative was founded in Denmark in 1936. The first yr more than 1,000 cows were artificially inseminated. The formation of farmer-owned artificial insemination cooperatives (Coops) in the United States came about after Professor Enos Perry, Rutgers University Extension Service, had earlier observed similar organizations in Denmark. Commercial AI service cooperative was made available to cattle producers in the United States in 1938. The first yr 1,050 cows were artificially inseminated. In the early 1940s, fresh bull semen was transported to dairy farmers for AI via a parachute in the USA.

The application of AI to aquatic species has been primarily directed toward aquaculture production and species preservation (Graham 1984). Until recently, the need to consider multiple sperm harvests from a single male has been minimal. Identification of genetically superior males requires multiple sperm harvests. The first harvest is made to obtain the offspring for progeny evaluation and subsequent harvests are used to disseminate the genes of the superior males in the breeding population. If only one ejaculate is available for both evaluation and dissemination, individual superior sires would not have sufficient impact on genetic improvement of a population to warrant this effort. Therefore, if fish males are to be used like bulls, males must be available for both aspects of the genetic improvement procedure. A procedure must be perfected to harvest semen without killing the male. In other words, don't kill the messenger before we get the message.

Figure 1. Leading Bess to the bull (adapted from Herman 1981).

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Extender Solutions

For AI to work effectively, yr of scientific research was conducted so that one ejaculate could be diluted or extended for use in breeding multiple cows. Semen has been extended in a variety of media including phosphate, Tris, citrate or other buffers as a base. Various additives have been included in the extenders such as coconut milk, tomato juice, caproic acid, egg yolk or other natural or synthetic materials (Foote 1978, Graham 1978). Milk has also been used as the entire extender in various forms such as skimmed, low fat, whole homogenized, evaporated, dried or buttermilk. Usually, a heating step is involved in milk extenders to destroy spermicidal enzymes, lactenin. Extensive reviews have been written on extender composition for cattle (Herman 1981, Herman et al. 1994, Pickett and Berndtson 1978; and others). The major component of all these extenders is water. Water serves to increase the volume and thus, the number of breeding units produced from a single sperm harvest.

The most commonly used extenders today are egg yolk-sodium-citrate-glycerol, egg yolk-Tris-glycerol, and milk-glycerol. Some semen processors use 7% (v/v) glycerol and others use 9% in the egg yolk-citrate extender; and these are generally used as a two-step extender. The two steps involve dividing the total extender volume equally, one fraction with glycerol and the other without. The non-glycerol fraction is usually added at 37 °C and the glycerol-containing fraction at 5 °C. The Tris extender is used with 7% (v/v) glycerol. However, sometimes it is used as a one step (25 °C) extender, yet other times used in two steps, one added at 37 °C and the other at 5 °C. The milk-based extender most commonly used is made with 3.5% homogenized whole milk and used as a two-step extender similar the yolk-citrate extender (Foote 1978, Graham 1978).

Sperm Storage Temperature

It has been shown that the modification of the sperm metabolism helps preserve the fertile life of bovine semen. It is well known that reduction of temperature can slow the rate of chemical reactions. Therefore, the simplest and most successful approach used to preserve sperm viability has been to decrease the storage temperature. Another method used is the principle of end-product inhibition. This has been achieved by gassing the semen with carbon dioxide, thus preventing the semen from producing carbon dioxide in the Kreb's metabolic pathways.

Ambient Temperatures

Early studies lead to the idea that adding antibiotics and gassing would prolong sperm life in the laboratory. Combinations of research efforts then led to what was known as the Illioni ambient temperature diluent, which was used to keep the sperm viable for up to and even greater than 7 d. This extender has been used extensively in swine artificial insemination, but is not used today in the cattle semen industry. Another extender, where these principles were used, was in the CUE extender that had high levels of bicarbonate as a potential carbon dioxide source, thus eliminating the gassing part of the procedure (Foote 1978).

Near Freezing or Cooled (5 ℃)

Chilled semen was used extensively in the early d of bovine AI. Unique methods were devised to maintain these cool conditions during storage. However, diluted semen was usually discarded after 4 d of storage (when fertility began to decline). This perishability required the

development of unique delivery systems, one of which was to parachute drop the cooled semen from an airplane, which was subsequently abandoned because of the expense. Most of the original extenders used were based on an egg yolk-citrate mixture. The refrigerator temperature reduced bacterial growth, reduced semen metabolism and the egg yolk stabilized the sperm membrane and protected the sperm from cold shock. The majority of semen processors could achieve the necessary temperature change (37 °C to 5 °C) in 90 min or less.

Frozen (-79 °C) to Deep Frozen (-196 °C)

There was a substantial amount of effort made by researchers in attempt to freeze semen in the presence of large sugar molecules. These sugar moieties were used to protect the sperm from the osmotic effect of increased salt concentration as water turned to ice. Other osmotically active molecules were also evaluated. The first attempt at freezing sperm (frog) using sucrose dehydration was reported in 1938 (Luyet and Hodapp 1938).

Then in the late 1940s and the early 1950s, British workers reported that glycerol protected semen during freezing (Polge et al. 1949). Subsequently, research on bull sperm freezing methods rapidly expanded (Stewart 1951, Polge 1952). Initially, semen was frozen in an alcohol-dry ice bath to a temperature of -79 °C. Containers were built to accommodate this method but unfortunately the dry ice and the alcohol had to be replenished every few d. Electric refrigerator units were also used to maintain this temperature. Even with this new approach, semen quality was found to continue to decline even at this temperature, as did the subsequent fertility, although at a reduced rate than that obtained with the using of cooled (5 °C)

By the late 1950s, studies reported an increase in success rates when freezing bull semen in liquid gases, such as nitrogen, oxygen or argon. The semen was diluted in an egg yolk or milk-based extenders that included glycerol. It was then decided to use liquid nitrogen (-196 °C) during the procedure rather than liquid oxygen, because it was colder, more reactive, and less expensive than liquid argon. Modified containers to hold LN_2, similar to a Thermos® bottle, were constructed and became commercially available in 1959. Although the cattle artificial insemination industry in New Zealand remained using semen cooled to 5 °C extensively for a period of time (Graham 1984).

Freeze-dried sperm

Researchers in Russia and at the University of Minnesota reported that reduction of the water content below 6% using freeze drying would not yield viable sperm upon reconstitution for insemination (Graham 1984). It was originally proposed that the use of this sperm dehydration approach had virtually no use for AI. However, with the advent of intracytoplasmic sperm injection (ICSI), freeze-drying sperm would greatly reduce the cost of LN_2 storage. If this methodology could be developed, it could someday be an alternative method of preserving the germplasm of endangered aquatic and other species. This germplasm could be used to produce a breeding population of a species for dissemination into other animal populations. Endangered species germplasm repositories could then operate without the need for LN_2. In recent yr, some success has been reported using dehydrated sperm in producing embryos in mice, rabbits, and cattle.

Semen Packaging for AI

Before usage of frozen semen, there was no need to consider special storage containers for bull semen because it was either totally used or discarded if older than 4 d after collection. In the early d of commercial cattle AI, semen was distributed to the farms in glass test tubes from which the breeding technician would withdraw 1 mL of semen to inseminate each cow.

Glass Ampules

With the advent of successful frozen-thawed semen for cattle AI, the best method of packaging semen for storage and in-field use became very important to semen processors. Although frozen semen storage provided advantages to the industry it created a storage space problem. Originally, semen was diluted on a volumetric basis in the laboratory. It soon became obvious to the processors that dilution on a sperm concentration basis would standardize semen evaluations and bull fertility estimates. For example, if the average bull ejaculate contains a volume of 7 mL, and a standard dilution (using a 20:1 dilution) would produce 140 1-mL breeding doses. Whereas, the average dairy bull ejaculate contains 1 billion sperm per mL and with 20 million sperm per breeding dose, this would result on average of 350 1-mL insemination doses per ejaculate from that bull. The latter approach to sperm dilution then increased demand for cold storage facilities and made more frozen semen available for cattle producers.

The glass ampule that was in use by the pharmaceutical industry was initially considered suitable for the cattle AI industry. These original ampules were constructed of Pyrex glass to withstand the freezing and thawing process. Although ampules came in a variety of sizes, the majority of the industry adopted the use of the 1-mL ampule for bull semen. This was ‘a fit’ because the industry had been inseminating cows with 1 mL of semen containing a standardized sperm number. Subsequently, one AI organization adopted the use of a 0.5-mL ampule for cattle.

AI Straw System

This straw system was originally developed for AI by the Danish in the early 1940s, using standard soda straws as the first frozen semen package. The straw system offered several advantages over the glass ampule method, which included the storage of more semen units in the same cold space. Also, the straw system improved the efficiency of the AI process because more sperm survived the freezing and thawing process. This was due to a greater surface-to-volume ratio of the straw as compared with that of the glass ampules. French researchers improved the straw system in the 1950s by reducing the volume of the breeding dose to 0.5 mL. With the improved freezing efficiency of the 0.5 mL straw, the straw system then improved fertility rates. The straw also delivered the entire semen dose at insemination because the semen unit was also part of the delivery system. Other modifications to the straw system were attempted including the use of a 0.3-mL straw, and the 0.25-mL straw. None of these straw modifications improved on the 0.5-mL French system.

Most of the AI organizations in the United States eventually adopted the French straw as the packaging system of choice. In Europe, some organizations have adapted the 0.25-mL straw simply because of limited storage space and the cost of LN2. Some of these organizations produce large banks of semen for each bull and then slaughter the bull. This prevents the overuse of any specific sire, and when land and barn space is at a premium, storing the semen in LN_2 cheaper than the bull.

Cryoprotectants

Glycerol

British researchers reported on the protective effect of glycerol during the freezing of bull semen in 1952. Glycerol was added at 5 °C to bull semen diluted in egg yolk-citrate extender (Polge 1952). It is interesting to note that semen cryoprotectants have usually been tested without polymeric materials, like egg yolk or other large molecular weight compounds, such as bovine serum albumen. Considerable research has been conducted over the yr in the use of glycerol in freezing bull semen. The interactions among glycerol concentration, rate of addition, timing of addition, and freeze and thaw rates have all been subject to study.

Today, the bovine AI industry uses glycerol and the concentration is varied according to the composition of the extender. The processors using egg-yolk citrate extenders will place 7 to 9% (v/v) glycerol in their extenders, while those that use milk as a base will add 9 to 11% (v/v) in their extenders.

Others

Colligative compounds, such as DMSO, 1,2-propanediol, 1,3-propanediol, ethylene glycol and others have been used effectively to freeze semen, yet none have been evaluated without the presence of polymeric materials (Dalimata and Graham 1997). Egg yolk is the only polymeric material to have any effect in the absence of a colligative compound.

Equilibration

Many studies have shown an interaction between equilibration time and glycerol concentration when using the 0.5-mL straw. While some have recommended shorter equilibration times and other have recommended longer times, most agree that this is a necessary step in the cryopreservation of bull sperm. The equilibration times that are used across today's industry range from 30 min to 6 hr. There has been a great deal of fine-tuning in this aspect of the freezing procedure but most meet the needs of the individual AI companies.

Freezing Time

This aspect of the freezing process has changed with the advent of the different packaging systems over the yr. The glass ampule required a longer time than the straw system because of the larger volume and smaller surface area of the ampule. Correspondingly, the straw has a larger surface-to-volume ratio and allows a faster freezing rate. There has also been reported that there is an interaction that occurs between the time it takes to freeze semen and glycerol concentration. Robbins et al. (1976) have shown that optimum acrosome retention was obtained when the semen extender contained glycerol at a final concentration of 9% (v/v) and the freezing rate was -19 °C per min. Other research suggested that the freeze should be completed in 20 min, while others recommended a time of only 8 min. Generally, the time spent in the freezing process is related to the type and concentration of the extender being used in the process. Today, most organizations freeze semen at a rate of 16 to 20 °C per min. When the temperature within straws reaches between -100 °C and -135 °C, they plunge the semen-filled straw into the LN_2.

Thawing Temperature and Time

Both the thawing temperature and thawing time interact with the packaging system and extender composition used in the process. The original recommendation for thawing of the glass ampule was to immerse it in an ice water slurry (4 °C) for 3 to 4 min. At our institution (Chandler et al. 1983b) showed that a warm water thawing techniques with the glass ampule would yield better post-thaw sperm quality.

The 0.5-mL straw has been subjected to a multitude of freeze-thaw studies over the yr. Recommendations have ranged from cold water thawing, air thawing, pocket thawing and a warm water thawing (35 to 40 °C) (Chandler et al. 1980, 1983a). With the air-thaw technique, the straw is simply exposed to the ambient temperature. The pocket-thaw method consists of wrapping the straw in a paper towel and placing it in the shirt pocket. Our work (Chandler et al. 1983a) was the last definitive report on the effect of thaw temperature and time for the 0.5-mL French straw. A warm water thawing of the straw was shown to result in better post-thaw semen quality and higher fertility rates.

The AI industry, with exception of the New York industry, has adapted the use of a warm water thaw for the 0.5-mL straw. This recommendation is to plunge the straw into a 35 to 40 °C water for 30 sec. The New York organization has maintained the use what they call the ‘pocket thaw’ for frozen bull semen that they process.

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Sources of Variation in Cryopreservation

S. P. Leibo

Introduction

Cryopreservation of gametes and embryos permits the genetics of animals to be stored in the frozen state at -196 °C in liquid nitrogen (LN_2) for decades (Glenister et al. 1990, Leibo et al. 1994), almost certainly for centuries, and probably for millennia. The procedure of gamete cryopreservation has become increasingly important in various aspects of biological research (Sharp and Mobraaten 1997), as well as in the breeding of domestic and wild animals (Wildt et al. 1997), and even in human clinical practice to treat infertility (Friedler et al. 1988). The comparative aspects of cryopreservation as they apply specifically to spermatozoa of various species have been recently reviewed (Leibo and Bradley 1999). Consider a few examples of the impact of gamete cryopreservation. Although figures have not been compiled recently, in 1980 it was estimated that, internationally, more than 25 million cows per yr were artificially inseminated with cryopreserved bull spermatozoa (Iritani 1980). That same survey found that fewer but still very substantial numbers of other domestic species were also inseminated with cryopreserved spermatozoa. At present, ~200,000 cryopreserved bovine embryos are thawed and transferred annually around the world; ~ 50% of these develop into calves (Thibier 1997). Each yr in North America alone, more than 1,500 babies are conceived by transfer of cryopreserved human embryos (SART 1999). Because of the risk of AIDS transmission by artificial insemination of women with semen from infected sperm donors, various public health and medical organizations and institutes have stipulated that all human semen be quarantined in the form of cryopreserved samples for at least 6 mo (Linden and Centola 1997). This use of frozen spermatozoa permits retrospective verification that the sperm donor is not seropositive for human immunodeficiency virus (HIV). All of these procedures depend on efficient cryopreservation of animal gametes and embryos.

However, there are many steps required to cryopreserve animal gametes and embryos, any one of which may damage or destroy the cells or tissues. More than 45 yr ago, based on his experiments with microorganisms, Mazur (1963) derived a series of mathematical equations to describe the response of cells when exposed to subzero temperatures. Much more recently, derivatives of that basic mathematical model have been used to optimize methods to cryopreserve mouse oocytes (Karlsson et al. 1996). Because cryopreservation involves a sequence of steps, even if all steps but one have been performed correctly, a single error in that one step may lead to the total loss of viable cells. Briefly stated, cryopreservation of all animal cells comprises the following: 1) collection of cells or tissues, and estimation of cell numbers and viability; 2) suspension of cells in a solution of a cryoprotective additive (CPA), and transfer into a sample container; 3) cooling of cells to low subzero temperatures under conditions to assure almost total cell dehydration; 4) storage of cells in LN_2 at -196 °C to assure long-term stability; 5) warming of samples to physiological temperatures, and 6) removal of the CPA, and assays of cell function to determine survival and normality. Because each of these steps may alter or damage the cells or lead to lowered functional efficiency, it is appropriate to consider each of them as sources of variability in the cryopreservation of animal cells.

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Collection of Samples

Even at the first step of cryopreservation, as cells or tissues are being harvested, various aspects of the physical or chemical environment in which they are handled may reduce or alter their function. For example, the medium used to collect the cells must be either prepared completely from pure chemicals or must be purchased from a reliable supplier of biological solutions. If media are prepared "from scratch," the water used must be of the highest possible purity and must be monitored regularly. This is especially critical with gametes, because these specialized cells are known to be extremely sensitive to even low concentrations of contaminants (Bavister and Andrews 1988). Special care must be exercised to assure that the chemicals, especially macromolecular supplements, are not contaminated. For example, until recently, the synthetic macromolecule, polyvinyl pyrrolidone (PVP; molecular weight = 40,000 daltons), contained a low molecular weight contaminant that, unless removed by dialysis, was toxic to mouse embryos (Leibo 1976). Even an indicator such as phenol red added to the medium to assure the correct pH can contain a contaminant with steroid-like activity, which may alter the cells to be cryopreserved. Another source of variability may be the ambient temperature at which cells or tissues are harvested, a variable that may be unimportant for some types of cells, but is critical for others. For example, if bovine oocytes are collected at three different temperatures (25, 30, or 35 °C) and then fertilized *in vitro* and cultured, the resultant embryos will contain significantly different numbers of cells 9 d later. These differences in cell numbers influence the likelihood of full-term fetal development (Pollard et al. 1996). That is, a significant effect of temperature during a 1-hr period of cell collection may not be evident until many d after the cells have been harvested, cryopreserved and assayed.

The CPA solution is an especially important factor in cell cryopreservation. First, one must consider the basic salt solution itself. If it consists of an inorganic buffer (e.g. sodium or potassium phosphate at neutral pH), the pH will change significantly during freezing, either increasing or decreasing, depending on the original pH and buffer concentration (Van den Berg 1959). Many yr ago, it was discovered that zwitterionic buffers were superior to inorganic buffers for sperm freezing (Graham et al. 1972). Yet again, macromolecular supplements added to the CPA medium can contribute unknown and variable sources of contaminants. Although in a different context, Bavister (1995) discussed the variability that bovine serum albumin (BSA) or other biological macromolecules (as opposed to synthetic compounds) can introduce into biological solutions.

Even when care is taken during media preparation, there is always the possibility of error. Therefore, it is prudent to perform some assay of the CPA medium prior to its use. Examples of such assays are measurement of the freezing point or osmotic pressure of the solution, or determination of its refractive index. When a CPA solution has been correctly prepared, there is the question of its storage until use. One alternative is to prepare a fresh solution every time it is to be used; another is to store the solution and to use it repeatedly over a long time. However, it is usually not desirable to store prepared solutions in standard freezers at -20 °C. Such freezers are usually "frost-free," which function by warming to ~ -12 °C and then re-cooling to -20 °C once every 24 hr. The relevance of this is that the eutectic temperature of most CPA solutions (the temperature above which the solution is not completely frozen) is -50 °C or lower; above the eutectic temperature, the solution is only partially frozen. This repetitive cycling subjects stored specimens to repeated melting and re-freezing. If CPA solutions are to be stored, it is best to

keep them at -75 °C or below. Finally, although not important for all types of CPA solutions, care should also be taken to assure that the media are sterilized before use. Yet even the method of sterilization (e.g. by vacuum filtration or autoclaving) must be considered carefully, so that essential components of the solution are not altered or removed.

Sample Containers

A variety of containers are available for the freezing of biological specimens. These containers may be made from glass or plastic, are available in many sizes, and some types may be purchased already sterilized. Freezing ampoules made of glass have been used for decades; they have the advantage of being non-reactive, are easy to sterilize if necessary, and are made to contain volumes of from 1 to 50 mL. However, glass ampoules must be heated to effect a hermetic seal. If the seal is not absolutely airtight, LN_2 may gradually seep into the ampoule, causing it to explode when it is warmed. For that reason, many people prefer to use plastic freezing vials to avoid the risk of injury that might be caused by explosion of glass ampoules. Plastic freezing vials are also available in various sizes from 1 to 5 mL, and have various shapes (e.g. round-bottom, cone-bottom, skirted to stand upright, or plain). In addition, the composition of plastic used by different manufacturers may vary. All of these physical factors of the vials affect their heat conductivity. Therefore, although it may be possible to cool samples in plastic vials at a low rate of a few degrees per min or less, it is not possible to warm such plastic ampoules at high rates of hundreds of degrees per min. But cell survival is as critically dependent on the warming rate as on the cooling rate (Leibo 1976, 1988, Mazur 1984, 1990, Rall 1992, Hochi et al. 1996). In other words, simply changing the type of freezing ampoule, or even the manufacturer of the same type, may influence the survival of frozen samples because the cooling and warming rates achieved with one type of freezing ampoule may differ from those yielded by a different type.

Small plastic straws were first used for freezing semen specimens by H. C. Adler as early as 1960 (Pickett and Berndtson 1974). Since then, they have been used to freeze spermatozoa from a very wide variety of laboratory, domestic, and wild animals, as well as humans. Plastic straws are now routinely used to freeze literally millions of samples of bovine semen for commercial breeding of cattle, especially in the dairy industry (Foote 1981). In 1978, they were first used to cryopreserve animal embryos (Utsumi and Yuhara 1975). Plastic straws are made to contain different volumes, from ~ 0.25 mL to 0.50 mL, up to 5 mL. Because of their cylindrical shape, straws have a high surface area to volume ratio; this means that they can be cooled from ~0 °C to -196 °C at rates of ~ 1,000 °C per min, a rate impossible to achieve with thick-walled plastic ampoules, even if the ampoules are immersed directly into LN_2 or a dry ice-acetone slush. They can also be warmed much more rapidly than plastic ampoules. If survival of the specific type of cell requires rapid warming, plastic straws are definitely the sample container of choice. Plastic straws are now used routinely to cryopreserve animal and human embryos. They are especially convenient for the cryopreservation of cattle embryos, because a single embryo can be frozen in one straw, permitting individual embryos to be thawed and transferred into recipient animals as they reach an appropriate stage of their estrous cycle (Voelkel and Hu 1992, Hasler et al. 1995, Dochi et al. 1995).

Although plastic straws have many advantages, they also have certain disadvantages that may introduce variability into gamete cryopreservation. For example, for certain applications, the straws must be sterilized. If autoclaved, the properties of the plastic are changed and the

solutions may not fill the straws properly. If straws are sterilized by ethylene oxide, the gas is absorbed by the plastic, and it takes a long time for the straws to "degas." If they are used too soon after sterilization, ethylene oxide will leach from the plastic into the solution containing the cells, a situation shown to be cytotoxic (Schiewe et al. 1988). Another potential disadvantage of freezing samples in plastic straws follows from the same characteristic that is an advantage. That is, when straws are removed from LN_2, they warm rapidly. That is an advantage when one wishes to thaw a sample. But it is a disadvantage if the frozen sample is held briefly in air without deliberately intending to thaw the sample. The sample may warm to as high as -50 °C or even -20 °C within only 10 to 15 sec. If the sample is then directly immersed again into LN_2, the cells may be killed by the sec rapid freezing. And this effect may not be discovered for mo or yr after the unintended thawing and re-freezing. All of these consequences depend, of course, on how the samples were frozen and may be influenced by the apparatus used for cooling.

Cooling Apparatuses

There are many types of instruments that have been developed to produce controlled-rate cooling. These range from simple metal blocks or glass chambers that are immersed in LN_2 and allowed to cool by conductivity to various electronic devices that cool a chamber either by mechanical refrigeration or by pumping tempered gas into the chamber. Each has advantages, but if not used properly, each can introduce variability from sample to sample. For example, simple chambers that are cooled by conduction by being placed into LN_2 have the advantage that they may not require electricity to operate and may be well suited for field use. However, if used under field conditions where the ambient temperature of the air may vary substantially (e.g. from 0 °C to > 30 °C), the chamber itself may be subject to some temperature variation. Cooling apparatuses that are electronically programmed may be subject to sudden cooling or warming if there should be a power loss, resulting in an interruption of the program. Those chambers that cool by refrigeration may have the advantage that they are relatively unaffected by a brief loss of electrical power. The most common type of cooling apparatus uses LN_2 as a coolant. The LN_2 is "tempered" (i.e. the liquified nitrogen is gasified), the gas itself is warmed to an appropriate temperature and pumped into the cooling chamber. These chambers are extremely efficient and reliable. One disadvantage to their use, however, is that they consume the same amount of LN_2 to cool a single sample as to cool many samples. A potential source of variability with this type of freezer is that the samples are cooled with cold gas. Because gas conducts heat poorly, samples nominally cooled at high rates may not actually be cooled at the programmed rates, despite the temperature reading indicated for the sample chamber.

The Cooling Method

An important potential source of variability is the cooling method itself. Stated simply, there are two general approaches to cell cryopreservation. These are referred to as Equilibrium and Non-equilibrium Cooling, and have been described in detail (Leibo 1989, Mazur 1990). Equilibrium cooling means that the cells are first allowed to reach osmotic and concentration equilibrium with the CPA. They are then cooled to a subzero temperature just below the freezing point of the CPA solution, and then ice is deliberately made to form, a phenomenon called "seeding" (Leibo 1990). Since most CPA solutions are used at concentrations of about 1.0 to 1.5 M, their freezing points are about -3 °C to -5 °C. Seeding removes water in the form of

crystalline ice, causing a concomitant increase in the CPA concentration. The cells are then allowed to equilibrate with this increased CPA, and then are cooled slowly to lower subzero temperatures. If multiple samples being cooled simultaneously are not seeded, the samples may exhibit significantly different survivals because ice will form spontaneously at very different temperatures even in identical samples. Seeding eliminates the randomness of ice formation. Once ice has formed in samples, the cooling must be slow enough to permit the cells to remain in osmotic equilibrium as the temperature decreases. This entire process has been modeled mathematically and has been shown to describe the response of cells as diverse as microorganisms and mammalian embryos (Mazur 1963, 1990). The key to the success of this cooling method is that the cells are allowed to remain in equilibrium with their physical and chemical environment during the entire procedure.

In contrast, cells can also be cryopreserved by non-equilibrium cooling. The first most successful application of non-equilibrium cooling to animal cells was the vitrification of mouse embryos (Rall and Fahy 1985). Vitrification refers to the fact that the aqueous solution in which the cells are suspended does not freeze, i.e. it does not crystallize. Rather, it forms a glass, or vitrifies. This is achieved by altering the solution in two ways. First, the concentration of CPA is increased substantially; sec, one or two macromolecules, such as PVP or Ficoll, are added to increase the viscosity of the solution. When this viscous solution containing 30 to 60% CPA is cooled rapidly, it cannot freeze. The fundamental and applied aspects of vitrification to cryopreserve various types of cells have been discussed in detail (Rall 1987, 1992). There are two potential sources of variability when cells are cryopreserved by non-equilibrium cooling. First, the cells must not be exposed too long to the CPA before being cooled; otherwise, the cells may undergo intracellular ice formation during rapid cooling. Even if exposed to the CPA just long enough, and even if cooled at the correct high rate, cells cooled by non-equilibrium cooling may be killed during the warming process. If not warmed rapidly enough, the vitreous water within the cells may crystallize (form ice) during warming. This “freezing during warming” may also kill cells.

“Plunge” Temperatures and Warming Rates

Another potential source of variability is the intermediate subzero temperature at which samples are “plunged” into LN_2. Most current methods of cell preservation stipulate that samples should be transferred from a slow cooling bath into LN_2 at a temperature of between about -30 °C to -75 °C. More than 20 yr ago, it was found that slow cooling of cells may be interrupted by rapid cooling at rather high subzero temperatures, but samples cooled this way must be warmed relatively rapidly if the cells are to survive. Many studies have been conducted to search for a single optimum “plunge” temperature. In fact, because populations of cells do not respond to freezing in an absolutely homogeneous fashion, such a single optimum temperature does not exist (Leibo et al. 1984). In general, cells plunged into LN_2 from high subzero temperatures (>-40 °C) must be warmed rapidly. Cells plunged into LN_2 from low subzero temperatures (<-65 °C) exhibit high survival if warmed relatively slowly.

Removal of CPAs from Cells

The last critical step of cell cryopreservation is the removal of the cryoprotectant from the cells. Performing this removal without damaging the cells depends on understanding the

phenomenon of cell permeability to water and to solutes. For many yr, it was thought that CPAs were toxic to cells, and that it was necessary to remove the cells from the CPA solution as rapidly as possible. This was usually done immediately after the suspension was thawed by diluting the CPA solution in a single abrupt step, sometimes when the suspension was still cold. Even when the cells had survived the freezing and thawing steps, this rapid dilution of the CPA often was sufficient to cause lysis of the cells by rapid influx of water. This rapid hydration of the cells caused an osmotic shock.

It is clear that it is preferable, and usually essential, for the osmotic shock to be avoided. There are several ways by which this can be achieved. The CPA can be diluted slowly by a gradual, step-wise dilution of the freezing solution, or by the use of a CPA to which the cells are extremely permeable, or by use of a so-called "osmotic buffer." The slow, stepwise dilution is easy to perform, but it is tedious and time-consuming. The alternative of using a compound to which the cell is more permeable requires specific information about the cell type and its permeability to various CPAs. The third alternative is to use an osmotic buffer. This is a solution of a low molecular weight, non-permeating compound, such as a monosaccharide or disaccharide. This solution is usually prepared at a concentration that is isosmolal to the CPA solution. That is, the osmolality of the osmotic buffer is equal to that of the CPA solution. When thawed cells containing a high intracellular concentration of CPA are placed into an isosmolal osmotic buffer (which contains no CPA), the CPA rapidly flows out of the cells because of the concentration gradient. However, because of the presence of the impermeant sugar, there is no difference in the osmolalities between the intracellular and extracellular solutions. Consequently, there is no net influx of water into the cell, no osmotic shock, and the cell easily survives removal of the CPA. The use of osmotic buffers to recover cryopreserved embryos from CPA solutions was first described by Leibo and Mazur (1978), and the principles of osmotic buffers and the use of stepwise dilutions have been described in detail (Leibo 1984, Schneider and Mazur 1984). The alternative of using CPAs to which cells are very permeable were first described for embryos by Rall et al. (1984), and by Renard and Babinet (1984). A variation of the osmotic buffer method has also been described by Massip and Van der Zwalmen (1984). Regardless of the specifics, it is important to recognize that the final step of cryopreservation, removal of the CPA, can be as critical to success as all of the other steps of the procedure.

Conclusions

Methods to cryopreserve gametes of many animal species are now very well established, and cryopreserved gametes have been used to propagate untold millions of living animals. This is unequivocal evidence of the success of these methods. Yet, because the methods involve a sequence of steps, there are many opportunities for errors. Careful attention to numerous small details can reduce or eliminate sources of variation in the cryopreservation of animal gametes and embryos.

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Annotated Bibliography of Developments in the Last Decade

Studies regarding the sources of variation in cryopreservation have an expanded since the first edition of this book in 2000. There have been more than five books and numerous papers published with content addressing variability in cryopreservation. There are many factors that determine the survival of cryopreserved cells including biological, chemical, and physical sources. The contribution of technical variation is fundamental to proper interpretation of this work and a complete understanding of the sources of variation is constrained by the lack of standardization in methods and reporting. For example, “male-to-male” variation could be caused by failing to address sperm concentrations during studies and, as such, a technical source of variation could be misinterpreted as a biological phenomenon. Future studies should focus on the controlling, refining, standardizing, and reporting of protocols.

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New Chapter

Non-Equilibrium Vitrification: an Introduction and Review of Studies Done in Fish

Rafael Cuevas-Uribe and Terrence R. Tiersch

The Need to Bank Aquatic Germplasm

Among vertebrates, fish represent more than 50% of all known species with a total of 28,800 described species (Diana 2003, Barton 2007). Of the total fish biodiversity, 43% of species are freshwater. Roughly 40% of the freshwater fish species in North America and European are imperiled (Jelks et al. 2008, Kottelat and Freyhof 2007). The main threats are habitat degradation and introduction of non-indigenous species. Overall declines are also occurring in the oceans. Since the 1970s, there has been an 80% decline in coral cover in the Caribbean. Coral reefs are among the most diverse ecosystems on Earth. About 35% of known fish fauna are associated with coral reefs (Barton 2007), yet reef fish density has been declining significantly for more than a decade, at rates ranging from 2.7% to 6% per yr (Paddack et al. 2009). In addition, large predatory fish biomass has been reduced by 90% over the last 50 yr (Myers and Worm 2003). Overfishing, pollution, global warming, ocean acidification, and other ecological impacts have degraded marine ecosystems (Jelks et al. 2008). Furthermore, according to the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN), 65% (940/1452 species) of the ray-finned fishes (Class Actinopterygii) that are listed as imperiled are less than 20 cm in body length. These small fish are typically overlooked in conservation programs. Given this dismal current reality, conservation efforts can no longer be delayed while awaiting more thorough assessments.

Cryopreservation in Fishes

Cryopreservation represents a tool for the protection of genetic resources in aquatic species, and offers many benefits in aquaculture, conservation biology, and medical applications. Benefits include the year-round availability of gametes, reduction of the need for maintaining fish as live populations, protection of valuable genetic lineages, and improvement of genetic lines (Tiersch et al. 2007). Despite these benefits, cryopreservation has only been researched for sperm of some 200 (Tiersch 2000) of the 28,800 fish species described (Barton 2007) with application only beginning, and research on eggs and embryos lagging behind this. As such, less than 1% of species could at present be preserved in “genome resource banks” or “frozen zoos”, with the vast majority remaining unstudied.

Cell Dehydration in Cryopreservation

The goal of cell preservation is to remove as much water as possible from inside the cell without disrupting its integrity (minimum critical volume) (Meryman 1974). The volume of water in almost all animal cells is 70-80%, except for erythrocytes (~50%), and spermatozoa (~50%) (Newton et al. 1999, Petrunkina 2007). There are several processes to dehydrate cells

Cuevas-Uribe, R. and T. R. Tiersch. 2011. Non-Equilibrium Vitrification: an Introduction and Review of Studies Done in Fish. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 309-324.

such as freeze drying (lyophilization) (Kusakabe et al. 2008), evaporative (convective) drying (Biggers 2009), vacuum drying (Meyers et al. 2009), exposure to hypertonic conditions for storage at room temperature (Van Thuan et al. 2005), or cryopreservation (Tiersch et al. 2007). Dehydration by exposure to hypertonic conditions during cryopreservation can be attained by cell exposure to cryoprotectants and extracellular ice crystals (Figure 1).

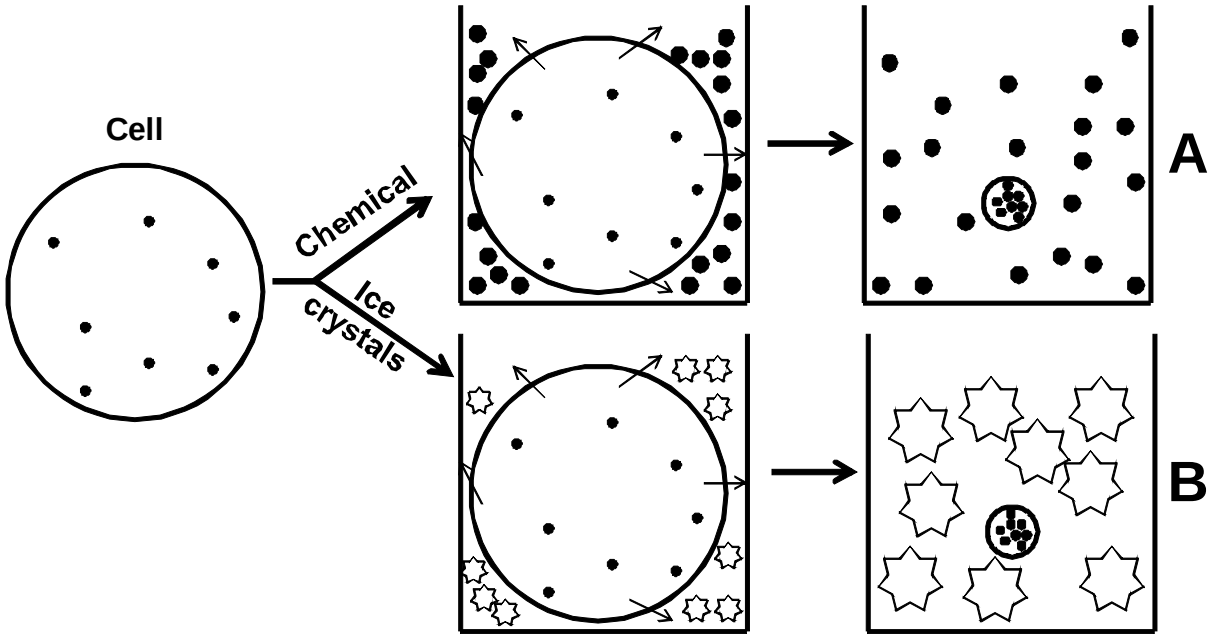

Figure 1. Because cells contain 70 to 80% water by volume, they can dehydrate by osmosis (arrows) until reaching a "minimum volume" known as the osmotically inactive fraction of cell volume. Hypertonic dehydration of the cell can be produced by: (A) non-permeating chemicals (●) and (B) ice crystals (✡). These mechanisms each act to increase osmolality of the extracellular space, which results in cellular dehydration, and thus reduces the chance of intracellular ice crystal formation. Water permeability depends on the cell type (size, membrane composition, shape, developmental stage), exposure temperature (permeability decreases at low temperatures), molecular size and charge (permeable or not), and concentration of molecules (diffusion gradients or differences in osmotic pressure). Note that there is not an increase in the amount of intracellular solutes, rather the cell reaches a minimum volume where the solutes are concentrated and thus the osmolality increases.

Exposure of cells to excessively hypertonic conditions can cause damage by osmotic stress and the solution effect (exposure to the effects of high solute concentrations, including low pH), and this type of injury is related to cell type, temperature, concentration of the suspending solution, and exposure time. The two factors that govern dehydration in cryopreservation are cryoprotectant concentration and cooling rate. When cooling rate is 'too slow', ice crystals will grow in the extracellular space, and the cells will be exposed for a longer period of time to a high hypertonic cold environment. Severe dehydration that leads to cellular disruption is a type of osmotic damage known as the 'solution effect'. When cooling rate is high, the cell will not have enough time to dehydrate because the ice crystals will grow quickly and the cell will become supercooled which will lead to the formation of intracellular ice (Figure 2, next page).

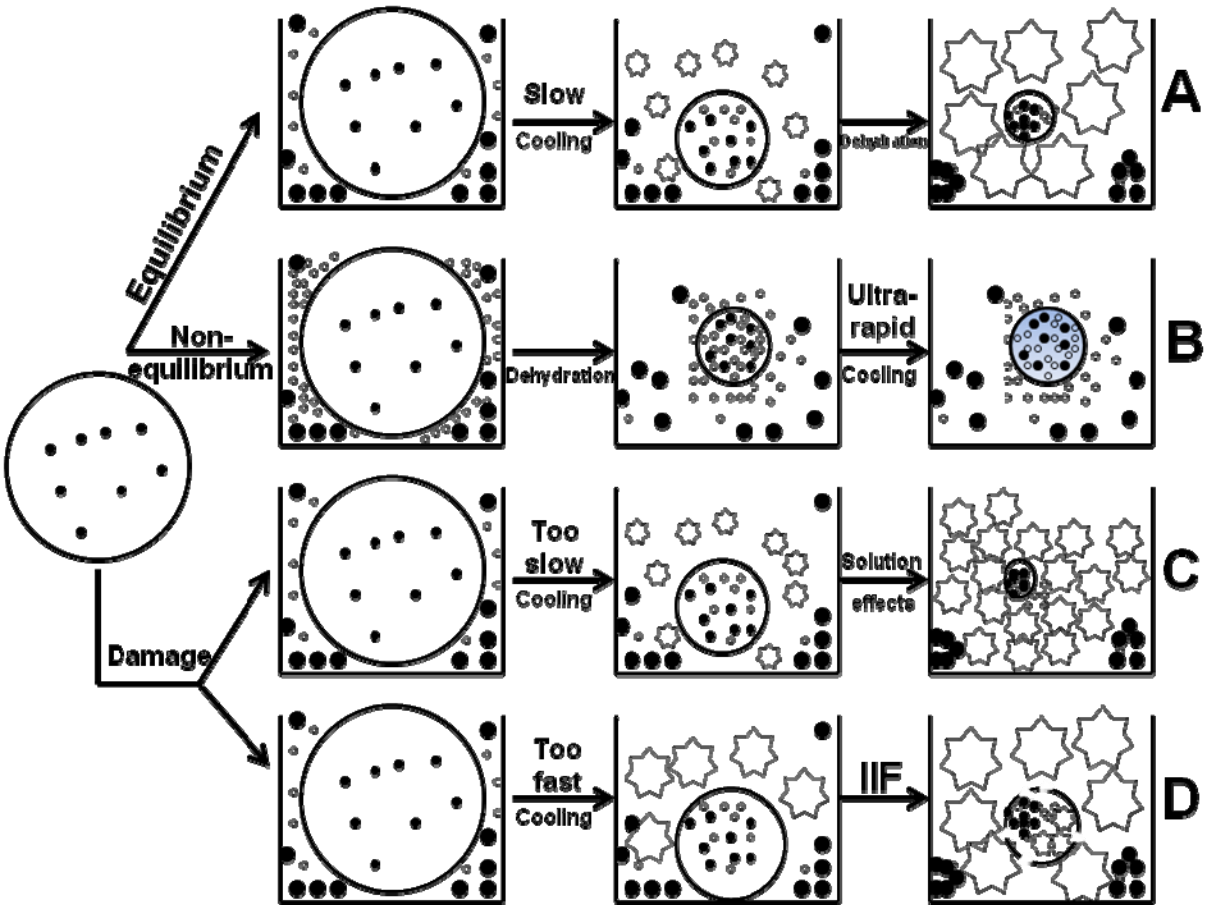

Figure 2. A) Equilibrium freezing involves the use of cryoprotectants and "slow freezing" to produce cellular dehydration and shrinkage. Permeating cryoprotectants (○) lower the freezing point of the solution, and minimize osmotic shock by replacing the water inside the cell. Non-permeating cryoprotectants (●) assist dehydration of the cell, and stabilize the membrane during cryopreservation. Once the first extracellular ice crystals (✡) have formed, as the temperature decreases, water is further incorporated into the growing ice crystals creating a hypertonic condition that produces osmotic dehydration. The combination of increased intracellular solutes and of lowering the temperature increases the viscosity of the solution until the eutectic temperature is reached (-40 °C) when the remaining unfrozen solution is solidified (partial vitrification). B) Non-equilibrium vitrification involves the use of high concentrations of cryoprotectants to dehydrate the cell and to replace the intracellular water before the cooling begins. Ultra-rapid cooling prevents the cells and the surrounding medium from undergoing ice crystal formation during cooling. The result is the solidification of the solution into a glass-like state (total vitrification). The two main sources of damage to the cell during cryopreservation are solution effect (C) and intracellular ice formation (IIF). C) Solution effect is due to excessively slow cooling rates, which cause the cell to experience severe volume shrinkage and long-term exposure to high solute concentrations. Excessive shrinkage can cause extrusion of membrane lipids and proteins, and intracellular changes such as reductions in pH that can denature proteins and cause loss of buffering capacity. D) Damage by intracellular ice formation is due to excessively fast cooling rates, which do not allow sufficient egress of intracellular water to maintain equilibrium and the residual supercooled water in the cell undergoes intracellular ice formation which causes damage by disruption of cellular structure and function.

Traditional cryopreservation seeks a happy medium between the time it takes for the cell to dehydrate without causing the solution effect, and a cooling rate that will not cause substantial intracellular ice formation. There is another procedure used in cryopreservation that consists of dehydrating the cell before cooling begins. This approach is known as 'rapid non-equilibrium vitrification'. This is different from slow equilibrium cooling protocols in that dehydration and cryoprotectant permeation take places before the cooling begins. In addition, the cells are exposed to an ultra-rapid cooling rate (typically >1,000°C/min) (Mazur et al. 1972, Leibo 1989, Mazur 2004). During this rapid cooling, the viscosity increases and the water molecules do not have time to arrange themselves into a crystalline structure, and therefore form an amorphous solid (vitrified) water. The resultant solid retains the random molecular arrangement of a liquid but has the mechanical properties of a solid (a "snapshot" of the liquid state). The temperature at which the sample is no longer a liquid but rather in the amorphous glassy solid (vitreous or non-crystalline ice), is known as the glass-transition temperature (-130 °C). The glass transition temperature can be raised by the addition of cryoprotectants (Fahy 1988). The purpose of vitrification is to reach the glass transition temperature as fast as possible through rapid cooling and by increasing the concentration of cryoprotectants. The goals of this chapter are to provide background on vitrification in general and to review the recent studies done in fish vitrification.

Equilibrium vs. Non-equilibrium Cryopreservation

Cryopreservation can be produced by two approaches: slow equilibrium cryopreservation (standard method) and rapid non-equilibrium vitrification. The main difference between these methods is that standard cryopreservation allows extracellular ice crystal formation while in vitrification ice crystal formation is suppressed. As stated above, dehydration of the cells during slow equilibrium freezing takes places during cooling, while dehydration in non-equilibrium vitrification takes places before cooling (Figure 2). To dehydrate the cell before cooling, high concentrations of cryoprotectants (40 – 60%) are normally used. To achieve the vitreous state before ice crystals have the chance to form (Kuleshova et al. 2007), it is necessary to rapidly cool through a specific temperature zone (-5 to -40 °C) (Shaw and Jones 2003) of potential crystallization. The resultant glass retains the random molecular arrangement of a liquid but has the mechanical properties of a solid (Taylor et al. 2004). This ultra-rapid cooling is typically done by plunging the samples directly into the liquid nitrogen (Fahy et al. 1984). Neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial (usually) or total intracellular vitrification can occur incidentally during traditional slow equilibrium cooling, and may help to ensure survival of some portion of cryopreserved samples (Vajta et al. 2009).

The goal of equilibrium freezing and non-equilibrium vitrification is to prevent intracellular ice crystal formation and to protect cells from damage. Vitrification is typically achieved by partial replacement of intracellular water via permeating cryoprotectants, which readily form glass, and by drawing out the intracellular water via non-permeating cryoprotectants (Figure 2). As a result, by combination of permeating and non-permeating cryoprotectants, the net concentration of the permeating cryoprotectant is increased in the intracellular space and their combined effect enhances the overall viscosity of the cell (Jain and Paulson 2006). In practice, the exposure to cryoprotectants is usually performed at room temperature (Kuleshova et al. 2007). The assessment of glass formation for cryoprotectants is relatively straightforward.

Crystallization can be distinguished by the observance of a milky appearance after plunging samples into liquid nitrogen, while glass formation appears as transparent (Figure 3) (Ali and Shelton 2007).

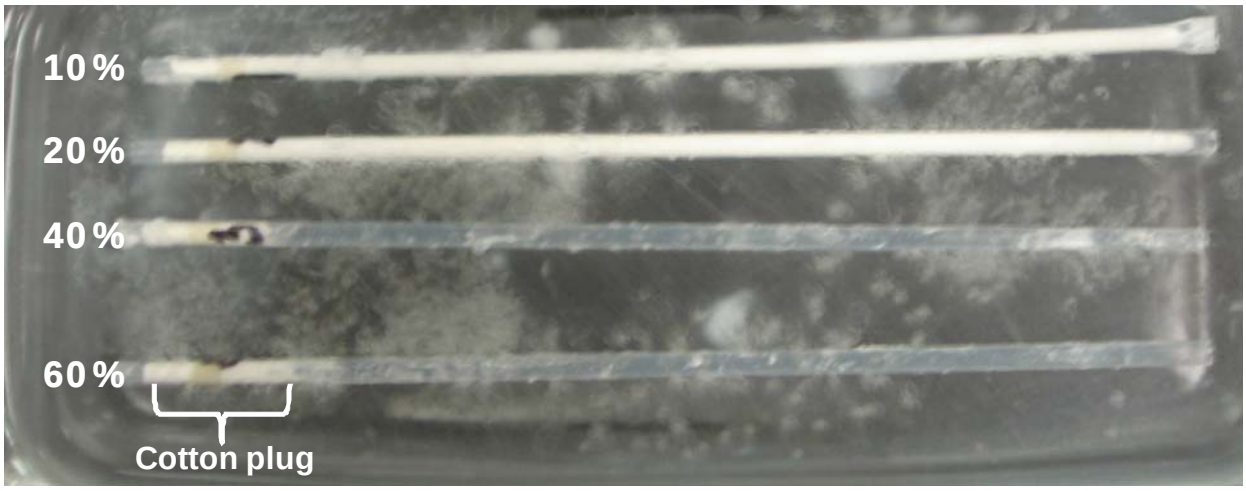

Figure 3. Visual observation of crystallization and vitrification of ethylene glycol at different concentrations. French straws (0.25-ml) with a cotton plug at one endwere filled with 10, 20, 40 or 60% of ethylene glycol and plunged into liquid nitrogen. Straws with 10 and 20% had a milky appearance indicating ice formation while straws with 40 and 60% remained transparent indicating glass formation.

Cryoprotectants in Vitrification

There are basically two types of cryoprotectants used in vitrification. The first type is the permeating cryoprotectants that are generally low molecular weight, non-electrolytes with high solubility in water. Permeating cryoprotectants have differential toxicity depending on the type, concentration, temperature, and time of exposure. The purpose of permeating cryoprotectants is to replace water from inside the cell with cryoprotectant. In this way the cell does not shrink beyond a minimum volume during cooling and these cryoprotectants decrease the freezing point while increasing the probability to form glass inside the cell. The rate of penetration depends on the chemical composition (e.g., molecular weight, hydrogen bonding capability, number and orientation of hydroxyl, amide, and sulfoxide groups) of the cryoprotectant as well as the properties of cell membranes (Leibo 2008). For vitrification purposes, each cryoprotectant forms a vitreous state at different concentrations. For example a strong glass former such as propylene glycol (MW = 76) produces glass at concentrations of ≥4 mol/L (30%), but methanol (MW = 32), which is one of the most permeable of cryoprotectants, is a weak glass former and will not vitrify even at high concentrations (crystallizes at 99.8%) (Ali and Shelton 2007). Due to the glass-formation properties and toxicity of the cryoprotectants to the cell, a mixture of cryoprotectants is often used for vitrification. Generally the mixture of cryoprotectants has a lower aggregate toxicity to the cell because it vitrifies at lower concentrations, and they combine the cumulative properties of each cryoprotectant such as permeability and glass formation.

The second type of cryoprotectants used in vitrification is known as the non-permeating cryoprotectants. The two main functions of these cryoprotectants are to dehydrate the cell during cooling by increasing the osmolality of the extracellular space, and to prevent excessive osmotic swelling during warming. Non-permeating cryoprotectants have high molecular weights (≥ 342 daltons) and can be monosaccharide sugars, disaccharide sugars, polysaccharides, and

macromolecules (Swain and Smith 2010). Adding sugars to the vitrification solution can increase the dehydration rate before cooling and enhance viscosity (Varghese et al. 2009). Sugars, especially disaccharides such as sucrose and trehalose, are effective in enhancing glass formation (Fuller 2004). Adding other agents such as polymers can facilitate vitrification and reduce the concentration of permeating cryoprotectants necessary to form glass (Fahy et al. 1984). Low molecular weight copolymers such as polyvinyl alcohol can inhibit ice formation and prevent the formation of ice crystals during warming (Wowk 2005). Another ice blocker is antifreeze proteins (AFP) that control the growth of ice crystals. Antifreeze proteins act by adsorbing to the surface of small ice crystals, inhibiting their growth. Antifreeze proteins could be used in vitrification to inhibit ice growth during warming (Fuller 2004).

Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification (Yavin 2007). There are a number of other ways to reduce the concentration of individual cryoprotectants required for vitrification, such as: applying high hydrostatic pressure, step-wise addition of cryoprotectants, and limiting exposure time at higher concentrations to a minimum (Fahy et al. 1984). In addition, the toxicity of cryoprotectants can be counteracted by the use of 'toxicity neutralizers' such as formamide or urea (Fahy 2010).

Devices Used in Vitrification

After the addition of cryoprotectants, the cells are ultra-rapidly cooled, usually performed in a single step in which the sample is plunged directly into liquid nitrogen. One hindrance to attaining rapid cooling by immersion in liquid nitrogen is the formation of a gas layer on the surface of the sample. Because liquid nitrogen is at its boiling point, heat withdrawn from the sample will vaporize the adjacent nitrogen, forming an insulating gaseous layer that retards the rate of heat transfer. But the rate of cooling can be increased by adding a thin insulation on the surface of the device (e.g., coating with talc) (Meryman 2007), or by application of a vacuum above the liquid nitrogen (nitrogen slush) (Shaw and Jones 2003, Yavin and Arav 2007). To achieve highest cooling rates and to prevent heterogeneous nucleation (formation of ice nuclei triggered by surfaces or impurities), the volume of the vitrification solution should be minimized (Dinnyes et al. 2007). To minimize the volume, special devices are used including: 0.25-ml French straws (45-µl sample volume, estimated cooling rate of 2,500 °C/min), 0.25-ml French straws (25-µl sample, 4,460 °C/min), open-pulled straw (1-µl sample, 16,700 °C/min), cryotop (0.1-µl sample, 23,000 °C/min), cryotip (1-µl sample, 12,000 °C/min), and hemi-straw (0.5-µl sample, 1,600 °C/min) (Chen and Yang 2007, Quinn 2010). In addition to cooling, the type of device used to vitrify influences the warming rate because its size and composition determine thermal conductance (Watson and Fuller 2001). Special attention is necessary when handling small-volume samples because of potential crystallization during storage or warming.

Sources of Variation in Vitrification

Some variables that influence the success of vitrification are: 1) the effects of exposure time to cryoprotectant solutions, and their concentration and temperature; 2) the number of steps in which the cryoprotectant is added and removed; 3) the type of device used for vitrification (which as stated above influences the size of the vapor coat and cooling rate); 4) the quality and the developmental stage of the cells tested (Liebermann et al. 2002); 5) the viscosity and volume of the sample (Yavin and Arav 2007); 6) the absolute pressure (higher hydrostatic pressures

decrease the homogeneous nucleation temperature and increase the glass transition temperature) (Rabin and Steif 2009), and 7) the warming process (ice crystal formation can occur during sub-optimal warming) (Leibo 2000). Overall, the high concentrations of cryoprotectants required are near the maximum tolerable limit of cells. As such, there is an inverse relationship between cooling rate and cryoprotectant concentration, i.e. the higher the cooling rate, the lower the concentration needed and *vice versa* (Mazur et al. 2008).

Thus to develop a vitrification protocol, the first step is to identify suitable vitrification solutions by measuring the toxicity of cryoprotectants at various concentrations, exposure times, and pre-freeze exposure temperatures. The second step is to select a vitrification device that will minimize the volume of the sample, and allow ultra-rapid cooling. Minimum volume methods allow the use of less concentrated cryoprotectants, and prevent heterogeneous ice formation (Vajta and Nagy 2006). The aim in any vitrification protocol is to increase the speed of temperature change while keeping the concentration of cryoprotectants (although high) as low as possible (Nawroth et al. 2005). Thus, vitrification should be performed in a kinetic way ('fast enough'), balancing concentration of the vitrificant and the rates of cooling and warming (Katkov et al. 2006).

Advantages of Vitrification

Vitrification is considered an attractive alternative to standard cryopreservation for specific applications and it has been used for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs in a variety of taxa (Tucker and Liebermann 2007). Vitrification does not require expensive equipment, is simple, achieves cryopreservation in sec, and can be used to preserve samples in the field. In addition, it offers perhaps the greatest potential for success in overcoming the challenges for preservation of fish eggs and embryos.

Vitrification of Fish Eggs and Embryos

Studies in fish vitrification can be dated back to 1938 when Basile Luyet attempted to vitrify juvenile goldfish *Carassius auratus* (40 mm, standard length) by plunging the fish into liquid air (-194°C) (Luyet 1938). Since then, there have been more than 30 publications dealing with embryo vitrification in 11 species of fish (most within the past 10 yr) (Table 1). Zebrafish *Danio rerio* is the most widely studied for vitrification, accounting for 50% of the studies. The results from vitrification studies had been controversial with documentation of "survival", but there has been a lack of reproducibility with these studies (e.g., Edashige et al. 2006). One of the main problems has been a lack of standardization in the methods and terminology used. For example, the term "survival" has been applied to "intact" embryos, hatched embryos, movement and twitching of embryos, and dye exclusion (such as trypan blue). Standardization of the terminology plays a key factor for reproducibility and validation. At least four studies have reported larvae hatched from vitrified embryos (Table 1), but none of these studies have been reproduced (Edashige et al. 2006). The limited success in fish embryo vitrification could be due to their large size (> 1 mm in diameter), low surface-to-volume ratio, the complexity of the multi-cellular embryo, and low permeability which could inhibit the entrance of cryoprotectants into the various embryonic compartments (Zhang 2004, Robles et al. 2009).

Table 1. Most studies on vitrification in fishes have occurred within the past 10 yr, and have addressed zebrafish embryos.

Species	Vitrified material	Summary of finding	Reference
Carassius auratus	40- mm fingerling	No survival	Luyet 1938
ND[a]	embryos	Intracellular blacking appeared	Wang et al. 1987
Clupea pallasi	eggs	Normal chorion and micropyles	Pillai et al. 1994
Clarias gariepinus	eggs	None survived	Magyary et al. 1995
Danio rerio	6 somite and heartbeat stages	≤ 32% intact morphology	Zhang and Rawson 1996
Danio rerio	Heartbeat stage	Two live embryos	Chao et al. 1997
Danio rerio	1 cell to prim-6 stages	≤ 80% intact morphology	Liu et al. 1998
Danio rerio	100% epiboly	All died	Hagedorn et al. 1998
Danio rerio	100% epiboly	Fell apart	Janik et al. 2000
Clarias gariepinus	eggs and embryos	No embryonic development	Urbanyi et al. 2000
Lateolabrax japonicus	neurula to prehatch	One embryo hatched	Tian et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 49% intact morphology	Robles et al. 2003
Scophthalmus maximus	tail bud and tail bud free	≤ 54% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	≤ 12% enzymatic activity	Robles et al. 2004
Danio rerio	high blastula and 5-somite stages	Differences of viable cells, SYBR[b]	Martinez et al. 2005
Pseudopleuronectes[c]	gastrula to starting pigment	1% continued development	Robles et al. 2005
Paralichthys olivaceus	20 somites to body movement	Seven embryos hatched	Zhao et al. 2005
Paralichthys olivaceus	neurula to hatching	Fourteen larvae hatched	Chen and Tian 2005
Paralichthys olivaceus	tail bud stage	No embryos survived	Edashige et al. 2006
Sparus aurata	tail bud and tail bud free stages	≤ 28% intact morphology	Cabrita et al. 2006
Danio rerio	caudal fin	≤ 63% attachment rate	Cardona-Costa et al. 2006
Danio rerio	blastomeres	≤ 20% survival, trypan blue	Cardona-Costa et al. 2007
Pagrus major	heartbeat stage	≤ 78% intact morphology	Ding et al. 2007
Prochilodus lineatus	morula to 6 somites	None viable	Ninhaus-Silveira et al. 2008
Carassius auratus	caudal fin	No cell outgrowth	Moritz and Labbe 2008
Danio rerio	5 somites	≤50% viable cells, SYBR	Martinez-Paramo et al. 2009
Tinca tinca	23 and 29 hr	No living embryos	El-Battawy and Linhart 2009
Danio rerio	testicular tissue	94% cell survival, trypan blue	Bono-Mestre et al. 2009
Danio rerio	blastomeres	90% survival and 20% recovery	Cardona-Costa et al. 2009
Danio rerio	64-cell to 20-somite stage	25% PGC[d] survival	Higaki et al. 2009
Danio rerio	14-20 somites	30% PGC survival	Higaki et al. 2010b
Danio rerio	14-18 somites	≤83% PGC survival	Higaki et al. 2010a
Danio rerio	stage III oocytes	≤69% survival, trypan blue	Guan et al. 2010

[a]ND: not described. [b]SYBR: SYBR-14 and propidium iodide. [c]*Pseudopleuronectes americanus*. [d]PGC: primordial germ cells.

Several approaches have been used to improve embryo vitrification in the studies listed in Table 1 by enhancing permeation of cryoprotectants. These include embryo dechorionation, enzymatic permeabilization (using the enzyme pronase), partial removal of yolk, microinjection of cryoprotectants, artificial expression of aquaporin-3, addition of AFP and polymers to inhibit ice formation and enhance glass formation, the use of cold-tolerant fish species (e.g., winter flounder *Pseudopleuronectes americanus* which produces AFP), assisted hatching techniques (e.g. piercing the egg), evaluation of different apparatuses and warming temperatures, and evaluating embryos at different developmental stages. Although some of these approaches have increased permeability, the concentrations necessary within the embryo for vitrification have not been achieved (Robles et al. 2009).

Future research on embryo vitrification could focus on neutralization of cryoprotectant toxicity (Fahy 2010), use of biopolymer-mediated intracellular sugars (Lynch et al. 2010), induction of suspended animation-like states before cooling (such as anhydrobiosis) (Blackstone et al. 2005), application of laser pulses (Kohli et al. 2007) and ultrasound (Wang et al. 2008, Silakes and Bart 2010) to increase permeability to cryoprotectants, and the use of innovative technologies such as magnetic field freezers (Kaku et al. 2010), and vacuum equilibration methods (Gwo et al. 2009).

New Strategies for Application of Cell Vitrification

Because cryopreservation of fish eggs and embryos has been unreliable, new technologies have been developed to conserve paternal and maternal genetic information. These technologies use surrogate production through transplantation of blastomeres, testicular cells (e.g., spermatogonial stem cells), or primordial germ cells (PGC) (Yamaha et al. 2007). Vitrification has been applied to cryopreserved blastomeres (Cardona-Costa and Garcia-Ximenez 2007, Cardona-Costa et al. 2009), testicular cells (Bono-Mestre et al. 2009), and PGC (Higaki et al. 2009, Higaki et al. 2010b) (Table 1). In fact, zebrafish (striped-type) were produced from surrogate zebrafish (golden-type, germ-line chimeras) that were generated through transplantation of vitrified germ cells (PGC from striped-type) (Higaki et al. 2010a). This is a breakthrough in cryopreservation because it presents alternative strategies to preserve fish genomes.

As part of biodiversity conservation strategies and to improve the genetic diversity through cryobanking of somatic tissues, vitrification has been applied to cryopreserve caudal fin cells from zebrafish (Cardona-Costa et al. 2006) and goldfish (Moritz and Labbe 2008). After cell culture of vitrified fin pieces, somatic cells were produced. Somatic cells should be considered for cryobanking of valuable or endangered fishes. In addition, somatic cells can be used to regenerate fish by nuclear transfer or somatic cloning technology (Siripattarapravat et al. 2009, Bail et al. 2010). Another method to regenerate fish is by androgenesis (all-paternal inheritance). Fertilization of irradiated eggs could be done by using: cryopreserved diploid sperm (e.g., from a tetraploid male) (e.g., Yasui et al. 2010), two cryopreserved sperm (dispermic androgenesis) (e.g., Grunina et al. 2006), or one cryopreserved sperm followed by suppression of first cleavage (Babiak et al. 2002). Another way to produce dispermic androgenesis that remains unexploited is by intracytoplasmic sperm injection (ICSI) of two cryopreserved sperm (Poleo et al. 2005a). ICSI has been used in fishes such as Nile tilapia *Oreochromis niloticus* (Poleo et al. 2005b), zebrafish (Poleo et al. 2008), and medaka *Oryzias latipes* (Otani et al. 2009).

Preservation of large sperm volumes is not necessary to reconstitute lines by production of founder populations. Vitrification could play a key role in the cryopreservation of small sperm volumes. Recently, vitrification of human spermatozoa was reported using small volumes (20 µL) (Nawroth et al. 2002). Several attempts have been made to plunge fish sperm samples into liquid nitrogen but none of these reports made specific reference to vitrification (e.g., Guest et al. 1976, Huang et al. 2004, Gwo et al. 2005). The combination of large volumes (> 250 µL), and low cryoprotectant concentration (<15%) make it unlikely that total vitrification occurred within these samples. Our recent studies have addressed development of generalized protocols for sperm vitrification in fish. Offspring were produced from vitrified sperm samples of channel catfish *Ictalurus punctatus*, green swordtail *Xiphophorus helleri*, and southern flounder *Paralichthys lethostigma* (Cuevas-Uribe et al. unpublished). From the recent work in sperm vitrification (our unpublished studies) it seems that marine fish sperm had higher survival after non-equilibrium vitrification, perhaps because they are adapted to deal with higher osmotic pressures (sea water > 1,000 mOsmol/Kg). Because the volumes used for sperm vitrification are small (~20 µL), this technique is currently best suited for use with aquarium fishes including endangered species. Sperm vitrification can be used to reconstitute lines from valuable biomedical models (such as zebrafish or *Xiphophorus* spp.), conserve mutants for development of novel lines for ornamental aquaculture, and transport frozen sperm from the field to the laboratory to expand the genetic resources available for germplasm repositories (Cuevas-Uribe and Tiersch unpublished).

Thus at present vitrification is most suited for use with microliter volumes of sperm, and other single-cell applications. It also offers potential for embryos and tissues if studies can be standardized and repeated among laboratories. More research needs to be done to evaluate the possible genetic consequences of using high concentrations of cryoprotectants (Tatone et al. 2010), and to evaluate the levels of microbial and viral contamination possible when samples are directly plunged into liquid nitrogen (Bielanski and Vajta 2009).

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New Chapter

The Use of Calorimetry for Subzero Cryobiological Measurements

Ram V. Devireddy

Overview

This article describes an advance in measurement methodology that has allowed direct assessment of subzero biophysical (freezing) response of small and irregularly shaped cells, such as sperm cells. It also describes the importance and the use of these measurements in developing rational sperm cell cryopreservation procedures.

Cryobiology Background

All cell systems share common cryobiological responses which can be exploited to better understand and alleviate the specific problems of freezing in reproductive (sperm) cells. In particular, when ice forms in a cellular suspension, electrolytes and proteins from the original extracellular solution are left within highly concentrated unfrozen liquid fractions. The cells, which remain unfrozen at the instant of ice formation within the extracellular space, respond to the increased concentration of the unfrozen fraction by either dehydrating (transporting water out into the unfrozen liquid fraction), or by the formation of intracellular ice in the cytoplasm (Mazur 1984). Dehydration is an attempt by the cell to reequilibrate the cytoplasmic concentration with the higher concentration extracellular unfrozen fraction by expulsion of cell water. Intracellular ice formation (IIF) occurs when super cooling (the temperature below the equilibrium phase change temperature) within the cytoplasm is sufficiently large to drive the nucleation and growth of an ice crystal within the cytoplasmic compartment.

Relationship Between Biophysics and Freezing Outcomes

As described above, the two important biophysical responses experienced by cells in the presence of extracellular ice during freezing are: a) water transport out of the cells (i.e., cellular dehydration) at 'slow' cooling rates, and b) intracellular ice formation (IIF) at 'fast' cooling rates. In the case of dehydration, the cell can be damaged by the increase of concentration both inside and outside of the cells as the temperature drops (predicted by the phase diagram of the solution) by a form of solute effects injury (Lovelock 1953). Intracellular ice formation (IIF) is generally considered lethal if more than 10-15% of the cellular water is involved (Mazur 1990). These two biophysical processes compete during any cooling procedure to dominate the freezing outcome. The highest rates of cellular survival are typically found for cooling rates which are ‘fast’ enough to minimize dehydration-induced solute effects injury while still ‘slow’ enough to preclude large amounts of intracellular ice.

This has been confirmed experimentally for a variety of cells, and the curve of cell survival plotted as a function of the cooling rate has a characteristic inverted U-shape (Mazur 1984). Whether a prescribed cooling rate is too 'slow' or too 'fast', is a function of cell membrane permeability to water and the probability that any water remaining trapped within the cell at any given subzero temperature will nucleate and turn to ice. Differences in membrane permeability to water and probability of IIF result in different 'optimal' cooling rates for different cells, for

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example in mouse embryos cooling rates of greater than 2 ˚C/min cause IIF, while in red blood cells the same occurs for cooling rates of greater than 8,500 ˚C/min. Thus, to optimize and generate a firm biophysical understanding of the freezing process in any biological system, both water transport (dehydration) and IIF need to be experimentally determined.

Measuring Subzero Biophysics

Water transport and IIF during freezing have been extensively studied in single spherical cells using cryomicroscopy techniques. Cryomicroscopy involves the application of cryogenic temperatures to cellular systems mounted under a light microscope to study the biophysical response of cells to freezing. Molisch (1897) first reported the study of living organisms (amoebae) under freezing conditions by placing the whole microscope in a cooled chamber. This technique has been refined and modified over the years to study the dynamic response of a variety of cells (plant and mammalian) under various freezing conditions (Diller 1982, Mazur 1984, McGrath 1988, Cosman et al. 1989, Toner et al. 1992, Devireddy et al. 1998, Berrada and Bischof 2001). Observations of these events can be fit to mathematical models which can be used to predict the cellular response to freezing under arbitrary cooling conditions, and hence help to develop better cryopreservation protocols. The central assumption in cryomicroscopy is that the projected two-dimensional area of the cell can be extrapolated to a spherical 3-dimensional volume, which is quite straightforward to do, if the cells are spherical. Unfortunately, the small radial dimensions, that are at or near the limits of light microscopic resolution (~ 0.2 μm), and the irregular (non-spherical) shapes of sperm cells do not allow observation and reduction of meaningful biophysical data through the cryomicroscope. Thus, none of the currently available cryomicroscopy techniques can be used to measure the biophysics during freezing in sperm.

Can Suprazero Biophysical Measurements be Extrapolated to Subzero Responses?

Nevertheless, due to the importance of the sperm cryopreservation question, many studies have been undertaken to characterize sperm water transport at suprazero (non-freezing) temperatures to attempt extrapolation to subzero dehydration behavior. Among the techniques that have been used are: 1) *Time to lysis* - this involves exposing cells to a hypotonic solution containing only non-permeating solutes to determine the time to lysis which is proportional to the permeability of the cell membrane to water (Curry et al. 1995, Gilmore et al. 1995, Noiles et al. 1993, Watson 1995); 2) *Coulter counter* - a particle counter and sizer can be used to measure volumetric changes in cell populations over time after initiation of an osmotic stress (Gilmore et al. 1995, Willoughby et al. 1996); 3) *Self-quenching of an entrapped fluorophore* - Spermatozoa can be exposed to hypo-osmotic and hyper-osmotic conditions using a stopped-flow apparatus and the time course of resulting volume changes measured using concentration-dependent self-quenching of the entrapped fluorophore, carboxyfluorescein (Curry et al. 2000, Chaviero et al. 2004). The parameters obtained using these suprazero techniques are essentially in agreement (Table 1); a suprazero membrane permeability to water (L_p) of 0.5 - 10 μm/min-atm and an activation energy at suprazero temperatures (E_a) of 3 - 15 kcal/mol serve to provide a working understanding of suprazero water (and cryoprotectant) transport response for sperm cells (Gao et al. 1997). It is interesting to note that these values for sperm cells, in general, when compared with other cell types are quite high for L_p and quite low for E_a.

Table 1. A listing of suprazero water transport parameters in mammalian sperm cells. Values of this type are not available for aquatic species. Values are listed in ascending value of L_p.

Species	L_p* (μm/min-atm)	E_a** (Kcal/mol)	References
Mouse	0.3 – 10.0	11.0 – 14.2	Willoughby et al. 1996; Noiles et al. 1997
Rabbit	0.63	17.8	Curry et al. 1994; Curry et al. 2000
Human	1.0 – 2.5	3.5 – 7.5	Noiles et al. 1993; Gilmore et al. 1995
Boar	8.47	1.06	Curry et al. 1995 Gilmore et al. 1998
Bovine	10.8	3.0	Watson et al. 1992

*Suprazero membrane permeability to water
**Activation energy or Temperature dependence of L_p

Comparing Apples to Oranges: The Perils of Extrapolation?

Extrapolation of the suprazero permeability data (obtained in the absence of extracellular ice using the techniques described above) to subzero temperatures (in the presence of extracellular ice) has not been successful. This conclusion is based on the fact that water permeabilities predicted on the basis of the above two approaches suggest that sperm cells should be able to dehydrate at rates as high as 10,000 ˚C/min during freezing when in fact experiments show that motility (a simple measure of post-thaw function) falls off when the cells are cooled faster than 100 ˚C/min (Henry et al. 1993, Noiles et al. 1993, Curry et al. 1994, Gao et al. 1997). Basically, the predicted 'optimal' rates of freezing for sperm cells are two orders of magnitude higher than those experimentally measured in the laboratory. Clearly, this is neither a meaningful nor useful result and has resulted in a long-standing dichotomy between experimentation and modeling in the field of sperm cryobiology.

Calorimetry as an Adjunct and Complementary Technique to Cryomicroscopy

It is clear that a technique that can obtain data on water transport (and IIF) during freezing would help explain this discrepancy and lead to a mechanistic understanding of how to optimize sperm cryopreservation protocols on a firm biophysical basis. To overcome the historical difficulties in obtaining biophysical data from sperm during freezing, a novel technique based on a differential scanning calorimeter (DSC) was developed by Devireddy and colleagues (1998). Data from the DSC technique showed that the initial heat release of a pre-nucleated sample containing osmotically active cells in media is *greater* than the final heat release of an identical sample of osmotically inactive or lysed cells in media (Figure 1, next page). The total integrated magnitude of this difference, Δq_{dsc}, was found to be proportional to the percent of volume occupied by live (osmotically active) cells, and hence also to the supercooled water volume in the sample. Further, the normalized fractional integrated heat release difference as a function of temperature, $\Delta q(T)_{dsc} / \Delta q_{dsc}$, was shown to correlate with the amount of supercooled

cellular water that had exosmosed from the cell as a function of subzero temperature at various cooling rates.

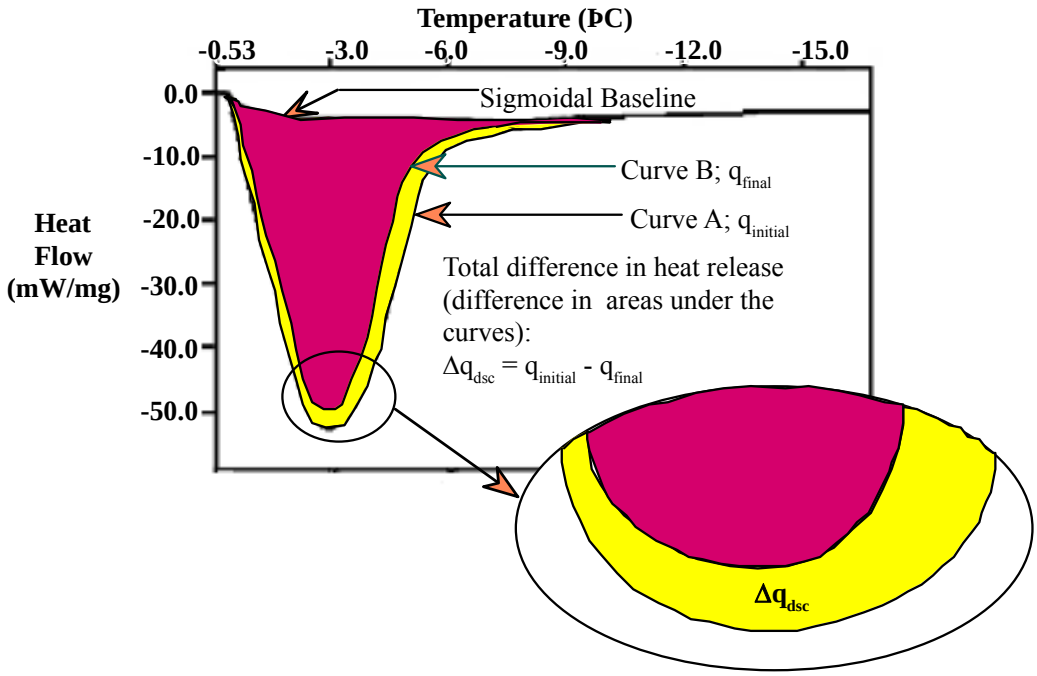

Figure 1. Superimposed heat flow thermograms measured by DSC obtained during the *initial* (Curve A) and *final* (Curve B) cooling runs. The negative axis for the heat flow on the y-axis implies an exothermic heat release in the DSC sample. The heat flow (mW/mg) is plotted along the y-axis and the subzero temperatures (˚C) are plotted along the upper x-axis.

The DSC technique was validated by showing that the measured water transport response for the cooling rate tested (5 ˚C/min) was statistically indistinguishable from that obtained by the traditional cryomicroscopy technique for a spherical cell (lymphocyte) system (Devireddy et al. 1998). Significantly, the DSC technique is independent of the size and shape of the cells being studied and is ideally suited to measure water transport response during freezing of irregularly shaped cells, especially for sperm cells. However, it is important to note some of the major limitations of the DSC technique are that: 1) it requires *a priori* knowledge of geometric parameters such as the surface area (A_c), initial volume (V_o), and osmotically inactive cell volume (V_b), 2) it alone cannot determine whether the heat released from supercooled cellular water is due to dehydration or intracellular ice formation, and 3) it cannot measure IIF. Cryomicroscopy or additional ancillary techniques and methods are needed to address these limitations.

Translation of DSC Measurements to Water Transport Response

The heat release measurements of interest are Δq_{dsc} and $\Delta q(T)_{dsc}$ which are the total and fractional differences between heat releases measured by integration of the heat flows during freezing of osmotically active (live) cells in media and during freezing of osmotically inactive

(dead) cells in media. The measured differences in heat release have been correlated to cell volume changes and consequently to water transport during freezing as: $\frac{V(T)}{V_o} = 1 - \frac{\Delta q(T)_{dsc}}{\Delta q_{dsc}} \times \left(1 - \frac{V_b}{V_o}\right)$, where V(T) is the sperm cell volume at temperature, T, V_o is the isotonic (initial) cell volume and V_b is the osmotically inactive cell volume.

Determining Membrane Permeability Parameters from the Water Transport Response: Model Description & Curve-Fitting Procedures

Water Transport Model

Kedem and Katchalsky proposed a model in 1958 for water and solute transport in response to chemical potential gradients based on irreversible thermodynamics. The Kedem and Katchalsky model consisted of two differential equations that described the water and cryoprotectant flux across the membrane (Kedem and Katchalsky 1958). If the flux of cryoprotectant is negligible in comparison to the flux of water (McCaa et al. 1991), the Kedem-Katchalsky model reduces to a model which assumes only water transport, as proposed by Mazur (1963) and later modified by Levin et al. (1976). The water transport model of Mazur was further modified to incorporate the presence of cryoprotectants on the volumetric shrinkage response of cells during freezing as (Karlsson et al. 1994): $\frac{V(T)}{V_o} = \frac{P_w \times A_c \times R \times T}{B}\left[\pi_i - \pi_o\right]$, with P_w, the subzero sperm cell membrane permeability to water defined by Levin et al. (1976) as: $P_w = fn\left(L_{pg}, E_{Lp}\right)$, where, L_{pg} and E_{Lp}, are the reference membrane permeability to water at 273.15 K and activation energy (or the temperature dependence of the cell membrane permeability), respectively; V(T) is the sperm cell volume at temperature, T; V_o is the isotonic (initial) cell volume; A_c is the effective membrane surface area for water transport, assumed to be constant during the freezing process; R is the universal gas constant; B is the constant cooling rate (˚C/min); π_i is the intracellular chemical potential and π_o is the extracellular chemical potential. In this model, every term is known *a priori* except L_{pg} and E_{Lp} which are determined as described below by curve-fitting the water transport model to experimentally obtained volumetric shrinkage data during freezing.

Numerical Methods

A nonlinear least squares curve-fitting technique is implemented using a custom-written computer program (Fortran 90) to calculate the membrane permeability parameters that best fit the volumetric shrinkage data as previously described by Bevington and Robinson (1992). The optimal fit to the experimental data is obtained by selecting a set of parameters which minimize the residual variance, and maximize a goodness-of-fit parameter. A goodness-of-fit value of 1.0 represents a ‘perfect’ fit while a value of 0.0 represents a completely ‘imperfect’ fit. Typically, the goodness-of-fit values are > 0.95.

Subzero Membrane Permeability Parameters for Various Sperm Cells

Over the last decade, measurements of the subzero membrane permeability parameters of sperm cell suspensions during freezing obtained with the DSC technique have been reported for mouse, human, equine, canine, boar and bovine (Table 2).

Table 2. Listing of subzero water transport parameters determined by DSC in mammalian sperm cells. Values are listed in ascending value of L_{pg}.

Species	L_{pg}* (μm/min-atm)	E_{Lp}** (Kcal/mol)	References
Canine	0.0027 – 0.0055	11.1 – 31.3	Thirumala et al. 2003
Mouse	0.004 – 0.01	12.1 – 50.6	Devireddy et al. 1999
Boar	0.0056 – 0.033	18.3 – 39.1	Devireddy et al. 2004
Equine	0.007 – 0.033	12.1 – 67.9	Devireddy et al. 2002
Bovine	0.02 – 0.036	26.4 – 42.1	Li et al. 2006
Human	0.04 – 0.22	31.5 – 93.5	Devireddy et al. 2000

*Subzero reference membrane permeability to water
**Activation energy or Temperature dependence of L_{pg}

Additionally, and of direct relevance to aquatic sperm cryopreservation, the subzero membrane permeability parameters of sperm cells from diploid and tetraploid Pacific oysters *Crassostrea gigas*, green swordtail *Xiphophorus helleri*, southern platyfish *Xiphophorus maculatus*, white bass *Morone chrysops* and striped bass *Morone saxatilis* have also been measured and reported (Table 3).

Table 3. Listing of subzero water transport parameters determined by DSC in aquatic species sperm cells. Values are listed in ascending value of L_{pg}.

Species	L_{pg}* (μm/min-atm)	E_{Lp}** (Kcal/mol)	References
Striped bass	0.001 – 0.016	8.8 – 40.2	Thirumala et al. 2006
Pacific oysters	0.0013 – 0.0028	7.1 – 14.8	He et al. 2004
Green swordtail	0.0024 – 0.0145	9.96 – 28.45	Thirumala et al. 2005
Southern platyfish	0.003 – 0.015	9.8 – 42.0	Pinisetty et al. 2005
White bass	0.003 – 0.01	20.0 – 31.4	Devireddy et al. 2006

*Subzero reference membrane permeability to water
**Activation energy or Temperature dependence of L_{pg}

Prediction of Optimal Cooling Rates Using Membrane Permeability Parameters

Recently, it was found that for a variety of biological systems the published experimentally determined values of optimal cooling rates, Bopt (in ˚C/min) agrees quite closely with the value obtained using a Generic Optimal Cooling Rate Equation (GOCRE) that is

defined as: $B_{opt} = 1009.5 \times \exp^{(-0.0546 \times E_{Lp})} \times (L_{pg}) \times (\frac{SA}{WV})$ (Thirumala and Devireddy 2005). In this equation, L_{pg} (in μm/min-atm) and E_{Lp} (in kcal/mol) represent the membrane permeability parameters, while the term SA/WV (in μm^{-1}) represents the ratio of the available surface area for water transport (SA = Ac) to the initial volume of intracellular water ($WV = V_o - V_b$). The use of GOCRE greatly simplifies the prediction of optimal freezing rates and is based on the assumption that the optimal rate of cryopreservation of any cellular system can be defined as the freezing rate at which 5% of the initial water volume is trapped inside the cells at –15˚C.

Reconciling the 'Dichotomy' Between Model Predictions and Experimental Observations

In general, DSC measurements have shown that subzero membrane permeability parameters in the presence of extracellular ice are significantly different than those obtained at suprazero temperatures in the absence of extracellular ice. In particular, the membrane permeability to water at subzero temperatures (L_{pg}) is lower by at least an order of magnitude than its counterpart at suprazero temperatures (L_p) while the activation energy at subzero temperatures (E_{Lp}) is higher by at least a factor of two than its counterpart at suprazero temperatures (E_a). This discrepancy between membrane permeabilities may be associated with possible changes in the sperm cell plasma membrane during suprazero cooling, including either a lipid phase transition between 0 and 4 ˚C (Noiles et al. 1995) or cold-shock damage or "chilling" injury during cooling (Watson 1981, Caffrey 1987).

The presence of extracellular ice further alters the cell membrane transport properties (McGrath 1988, Schwartz and Diller 1993). For example, the temperature reduction which induces solidification in the extracellular medium may lead to lyotropic (i.e., independent of cooling rate) membrane phase changes and corresponding alterations of membrane permeability (Steponkus 1984, Drobnis et al. 1993) and membrane fluidity (Blok et al. 1976). In general, for mammalian cells the average activation energy obtained in the presence of extracellular ice is approximately twice as large as that for studies conducted in unfrozen solutions at higher temperatures (Schwartz and Diller 1993). A direct consequence of this dramatic difference between the subzero and suprazero water transport parameters is that the optimal cooling rates predicted by utilizing the subzero parameters (tens of ˚C/min) are significantly lower than those obtained by using the suprazero parameters (thousands of ˚C/min). And more importantly, these subzero measurements were able to reconcile the previously described dichotomy between model predictions and experimental observations in sperm cryobiology. As a specific example, the permeability parameters obtained for striped bass sperm cells predict an optimal rate of cooling of ~14 ˚C/min, which is quite close to the experimentally determined value (Thirumala et al. 2006). This close agreement between the numerically predicted and experimentally determined optimal cooling rate is especially noteworthy considering the various assumptions made not only in the development of the water transport model but also in the development of the Generic Optimal Cooling Rate Equation, and suggests that the membrane permeability parameters obtained using the DSC technique can help to establish cryopreservation of sperm cells of aquatic species on a firm biophysical basis.

Future Calorimetric Measurements in Cryobiology

One of the major drawbacks of the calorimetric technique, as described above, is the inability to distinguish between the biophysical responses of water transport and IIF. Hence, all of the calorimetric experiments are performed with cooling rates that are presumably 'slow' enough to induce water transport only, typically less than 20 ˚C/min. In theory, experiments performed at 'fast' cooling rates should only induce IIF and it might be possible to deduce the probability parameters that govern the formation of intracellular ice in sperm cells from experimental data obtained at these 'fast' cooling rates (typically ≥ 100 ˚C/min). Unfortunately, the accuracy, repeatability and performance of the commercially available calorimeters are significantly reduced when the imposed cooling rates are greater than 40 ˚C/min. Thus, there is as yet no mechanism to utilize calorimetry to measure IIF in sperm cells. As our ability to measure temperature and control heating and cooling increases, it should be possible to construct calorimeters that are capable of imposing controlled cooling rates of hundreds of ˚C/min and allow direct assessment of IIF in sperm cells.

Final Thoughts: Where There is Smoke, is There Always a Fire?

Recent experimental evidence by Morris (2006) and Morris and colleagues (2007) suggests that the widely accepted two-factor (water transport and IIF) injury mechanism during freezing might not be valid for sperm cells. The lack of direct visual evidence for the formation of intracellular ice in cryo-scanning electron microscopy images of human sperm cooled as fast as 3000 ˚C/min led to a postulation that the cell damage during freezing and thawing is due to the osmotic imbalance during thawing (i.e., the damage to sperm cells is not during the freezing process but in the thawing process) (Morris 2006, Morris et al. 2007). Assuming that the extremely small intracellular ice crystals that could have formed during freezing are, indeed, amenable to direct visualization by electron microscopy techniques, these observations lead to the following questions: 1) Are sperm cells unique in their response to freezing injury? 2) Does the direct observation of IIF (and water transport) in other cells have any bearing on the outcome of their freezing process? Have we been looking at the smoke (biophysical response) and postulating the presence of a 'damaging' fire (the post-thaw outcome), or is there a need to re-evaluate the whole two-factor injury mechanism in other cells as well? 3) Do we need to re-evaluate the two-factor injury mechanism, which even if incorrect, does seem to predict the outcome of freezing process to a reasonable degree? And finally, 4) What new models and techniques are needed to further corroborate and predict the response of cells to freeze-thaw stress? These are some of the questions that current and future scientists, engineers, and biologists are beginning to address and only time will answer.

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IV. Cryopreservation of Sperm of Vertebrates

Preliminary Investigations on Sperm Cryopreservation of a Stingray, the Sparsely Spotted Stingaree

Jonathan Daly, Michael K. Holland and David B. Galloway

Introduction

Elasmobranchs, comprising sharks, skates, and rays, are held in aquaria for display, conservation, and educational purposes. A vast amount of work is being conducted into understanding the life history and population demography of wild populations in Australia, but relatively little is understood about breeding these species in captivity. Breeding programs traditionally rely on the occurrence of natural mating. While this approach has been successful in breeding many species of elasmobranch in aquaria (e.g., Henningsen et al. 2004), there are still many species that have never reproduced in captivity (e.g., *Notorynchus cepedianus*) while other species reproduce sporadically (e.g., *Carcharias taurus*). A wide range of factors contributes to successful natural breeding of elasmobranches in aquaria, including temperature cycles, population size and structure, and environmental considerations created by the need to cater for multiple species in a single tank or system. If conditions are not optimal for a particular species, potentially important breeding populations can become non-viable. In addition, females of many species of viviparous sharks have long reproductive cycles (Hamlett and Koob 1999, Daly et al. 2007), and if a breeding season is missed it could be years between successful matings. Even when successful breeding is achieved, loss of genetic diversity can occur due to low population size relative to that in the wild. Assisted reproductive technologies, such as cryopreservation and artificial insemination, have the potential to help overcome many of these problems. Access to cryopreserved sperm would allow greater control of breeding and genetic diversity of captive populations, reduce the need to supplement aquarium populations from the wild, and enable the establishment of gene banks for endangered species held in aquaria.

The aim of the present research was to develop a protocol that could be used as a starting point for cryopreservation of elasmobranch spermatozoa. Although experiments have been conducted on sperm activity *in vitro* (Minamikawa and Morisawa 1996), there are no reports of cryopreservation of elasmobranch spermatozoa in the literature. The sparsely spotted stingaree *Urolophus paucimaculatus*, a stingray, was the elasmobranch species of choice for this research because it is common and has a high occurrence as a bycatch species in local fisheries.

Materials and Methods

Sample Collection

Sperm production in this species is seasonal (Spring - Summer, September - February). Sparsely spotted stingarees were obtained as deceased by-catch from beach seine fishermen operating in Port Phillip Bay, Victoria, Australia, in February of 2008 and stored at 4 °C. Stingarees were dissected within 12 hr of collection. Sperm were collected directly from the ampullae of the vasa deferentia by inserting an 18-gauge needle attached to a 1-mL syringe. Individual samples were assessed visually under light microscope (× 400) by placing a small drop (1-2 μL) onto a glass slide and adding 10 μL of elasmobranch Ringer solution (defined

Daly, J., M. K. Holland and D. B. Galloway. 2011. Preliminary Investigations on Sperm Cryopreservation of a Stingray, the Sparsely Spotted Stingaree. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 337-344.

below). Samples showing strong activity (>80%) were pooled.

Composition of Base Media and Egg Yolk Preparation

Three base media were used in extender preparation. Elasmobranch Ringer (ER) solution (257 mM NaCl, 7 mM Na_2SO_4, 2.5 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 3 mM $MgSO_4$, 70 mM trimethylamine N-oxide, 0.27 mM Na_2HPO_4, 0.01 mM NaH_2PO_4, 400 mM urea, 30 mM glucose) was prepared according to Simpson and Sargent (1985). Tris-sucrose-potassium (TSK) solution (30 mM Tris, 25 mM sucrose, 600 mM KCl) was adapted from Horvath et al. (2005). And D-Sorbitol (DS) was prepared as a 1.2-M solution. All base media were prepared in filtered deionized water (Milli-Q, Millipore, Billerica, Massachusetts, USA) and adjusted to an osmolality of 1100 mOsmol/Kg and pH 7.5.

Yolk obtained from fresh chicken eggs (Clayton Egg Farm, Clayton South, Australia) was heated in a water bath at 56 °C for 30 min to inactivate microbiological agents. The yolk was allowed to cool to room temperature and added to the base media, and was clarified by centrifugation (Mikro 22 R centrifuge, Hettich Zentrifugen, Germany) at 15,000 × *g* for 30 min, followed by filtration through a 20-µm syringe filter (Minisart®, Sartorius Stedim Biotech, France).

Experiment 1

Sperm were diluted 1:9 with extenders, containing final concentrations of 0, 5, 10, 15, and 20% (v/v) egg yolk and 10% (v/v) dimethyl sulfoxide (DMSO) in ER. Aliquots of 100 µL were drawn into 0.25-ml French straws (IMV, France) using a micropipette. Straws were sealed with stainless steel balls and placed in a Freeze-Control® cryochamber (CryoLogic, Australia, *www.cryologic.com*) at an initial holding temperature of 20 °C, and frozen using a pre-existing program on the control unit as follows: straws were cooled from 20 °C to -6 °C at 3 °C/min, held at -6 °C for 5 min, cooled from -6 °C to -40 °C at 1 °C/min, from -40 °C to -160 °C at 3 °C/min, and plunged into liquid nitrogen (-196 °C) and stored until assessment.

Experiment 2

Three base media (ER, TSK, and DS) and three cryoprotectants (DMSO, glycerol, and methanol) were used in this experiment. Sperm were diluted 1:9 with extenders containing final concentrations of 20% egg yolk, 10% cryoprotectant, and 5 mM reduced glutathione. Aliquots of 100 µL were drawn into 0.25-ml straws and frozen as described above.

Thawing

Straws were thawed by gentle agitation in a water bath at 20 °C for 10 sec and immediately diluted for post-thaw assessment with 100 µL of the same base medium that was used for freezing.

Post-thaw Assessment of Sperm Quality

Sperm activity was estimated under a light microscope at 400-× magnification by placing a 10-µl aliquot onto a glass slide under a cover slip. Activity was estimated independently by two observers, and the average percentage of sperm showing activity was recorded. Observations were made within 1 min after thawing, and at 5, 15, and 25 min after thawing.

Sperm membrane integrity was assessed using a live/dead fluorescent staining protocol (SYBR 14/ propidium iodide [PI], Molecular Probes, Eugene OR) with adjustments. Samples

were stained with SYBR 14 (200 nM) and incubated in the dark at room temperature for 5 min. After this time, PI (2.4 μM) was added to all samples and incubated for another 5 min. Samples were centrifuged at 1000 × *g* for 1 min, the staining solution was removed, and the sperm pellet was re-suspended in 500 μL of ER. The centrifugation procedure was repeated, and sperm were re-suspended in 500 μL of ER. A total of 20,000 gated events (based on forward scatter vs. side scatter) were analyzed per sample using a flow cytometer (BDFACS Canto™ II, Becton Dickinson, USA) with 488-nm laser excitation. SYBR 14 was detected with a 530/30 nm band pass filter, and PI with a 670 nm long pass filter. Cell populations stained with SYBR 14 alone (membrane intact) were considered to be viable. Those stained with both SYBR 14 and PI or PI alone (membrane compromised) were considered to be non-viable.

Statistical Analyses

Percentage data were transformed to arcsine values for statistical analyses. One-way ANOVA followed by Tukey's multiple comparison t-test was performed using GraphPad Prism version 5.0 for Macintosh (GraphPad Software, San Diego California USA). Univariate analysis of variance to determine factor interactions was performed using SPSS for Macintosh (Version 12.0.1, SPSS Inc., USA).

Results

General Observations

Spermatozoa of sparsely spotted stingaree were characterized by a spiral head, a short midpiece, and a long thin filamentous tail (Figure 1). Sperm movement was characterized by rapid rotation of the head along its longitudinal axis (spiralling) with or without forward progression. As 'motility' (the usual term for sperm movement) implies rapid forward progression, it was decided that 'activity' was a more appropriate term. Maximum percent activity observed in fresh sperm during the study was 90%, the pooled sample used for cryopreservation had ~80% activity prior to freezing.

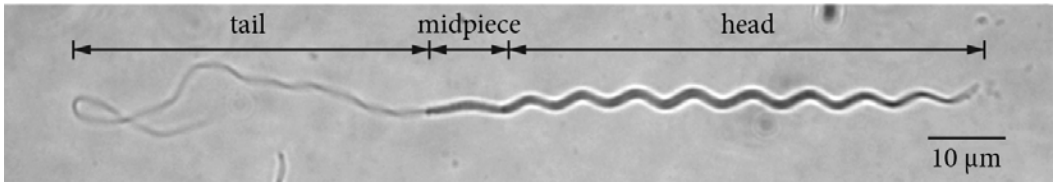

Figure 1. A sperm cell of the sparsely spotted stingaree. Activity was characterized by rapid spiralling of the head with or without forward movement.

Experiment 1

Egg yolk had a significant effect on post-thaw viability of spermatozoa ($P < 0.01$), with increasing egg yolk concentration in the extender corresponding to increasing post-thaw membrane integrity (Figure 2). The highest post-thaw membrane integrity was found when sperm were frozen in the presence of 20% egg yolk (23 ± 2% membrane intact), which was significantly better ($P < 0.01$) than all other treatments except 15% egg yolk (19 ± 1% membrane integrity). The effect of egg yolk on activity was less pronounced. There was a general but not

significant increase in post-thaw activity with increasing egg yolk concentration (Figure 2). Activity declined to less than 10% within 5 min of thawing in all treatments.

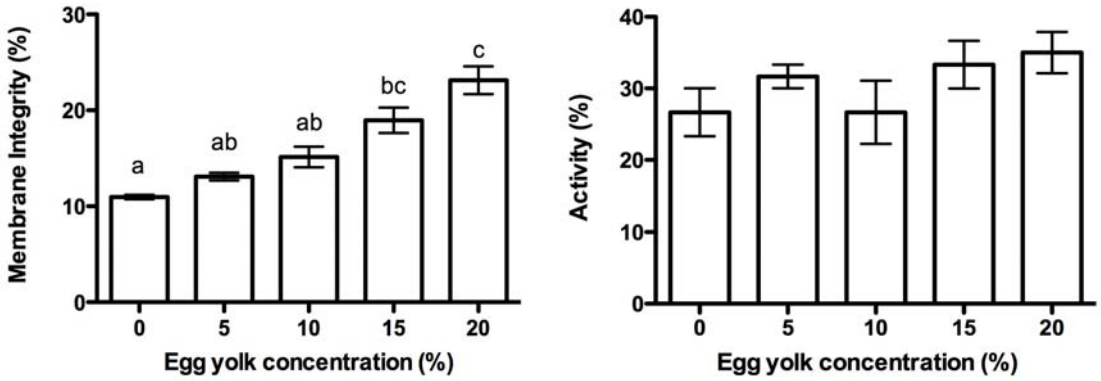

Figure 2. Effect of increasing egg yolk concentration on post-thaw membrane integrity (left panel) and initial post-thaw activity (right panel) of sparsely spotted stingaree sperm. Values sharing letters were not significantly different at $P < 0.01$ (there were no differences among the activity values). Values are the mean ± SEM of 6 samples.

Experiment 2

Cryoprotectant and base medium each had a significant effect on post-thaw membrane integrity ($P < 0.01$). The effect of the interaction between these two factors on post-thaw membrane integrity was also significant ($P < 0.01$). The highest post-thaw membrane integrity was observed when sperm were frozen in an extender composed of ER with 20% egg yolk and 10% glycerol (23 ± 0% membrane intact) which was significantly better than all other treatments ($P < 0.01$) (Figure 3). Glycerol was the most effective cryoprotectant tested, resulting in higher post-thaw membrane integrity than DMSO and methanol in all media. Methanol offered little cryoprotection to sperm in any media.

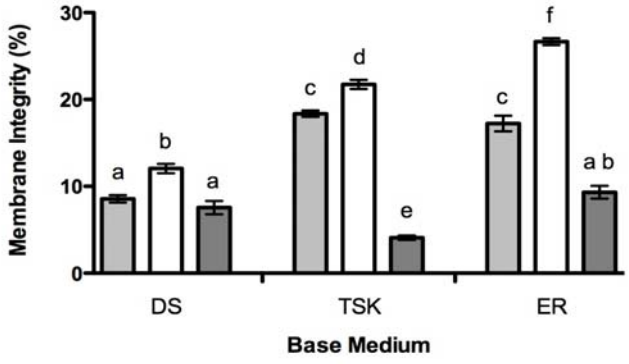

Figure 3. Post-thaw membrane integrity of sperm frozen in extenders containing DS, TSK, and ER with 20% egg yolk and 10% cryoprotectant. Key: DMSO □, Glycerol □, Methanol ■. Columns sharing letters were not significantly different at $P < 0.01$. Values are the mean ± SEM of 6 samples.

Sperm frozen in an extender composed of ER with 10% DMSO had the highest initial activity (38 ± 3% at 1 min after thawing), followed by TSK with 10% glycerol (36 ± 4%) (Table 1). The highest initial post-thaw activity (ER with 10% DMSO) was significantly better than the lowest (TSK with 10% methanol, 15 ± 3%) ($P < 0.01$), but there were no significant differences in initial post-thaw activity among any other treatments (Table 1). Sperm frozen in extenders containing TSK with DMSO or glycerol sustained post-thaw activity better than all other treatments (Table 1), while sperm declined rapidly in extenders using ER or DS as the base medium and in all extenders containing methanol (Table 1). At 25 min after thawing, the activity of sperm frozen in TSK with 10% glycerol (13 ± 2%) or TSK with 10% DMSO (10 ± 1%) was significantly better than all other treatments ($P < 0.01$) (Table 1).

Table 1. Percent activity of spermatozoa at four time points after thawing.

	Percent activity after thawing			
Treatment	**1 min**	**5 min**	**15 min**	**25 min**
DS + DMSO	23 ± 4	5 ± 0	0 ± 0	0 ± 0
DS + Glycerol	28 ± 3	8 ± 1	4 ± 1	0 ± 0
DS + Methanol	28 ± 2	8 ± 1	5 ± 2	3 ± 0
TSK + DMSO	31 ± 3	17 ± 1	13 ± 1	10 ± 1 *
TSK + Glycerol	36 ± 4 +	20 ± 1	13 ± 2	13 ± 2 *
TSK + Methanol	15 ± 3 +	7 ± 3	3 ± 3	3 ± 3
ER + DMSO	38 ± 3	9 ± 1	5 ± 1	1 ± 1
ER + Glycerol	33 ± 3	7 ± 2	3 ± 1	0 ± 0
ER + Methanol	32 ± 4	8 ± 1	3 ± 1	0 ± 0

Values are the mean ± SEM of 6 samples.
+ Indicates a significant difference between the two values ($P < 0.01$).
* Indicates a significant difference from other values in the same column ($P < 0.01$).

Discussion

To our knowledge, this is the first report of cryopreservation of spermatozoa from an elasmobranch species. In the present study, post-thaw activity and membrane integrity were lower than those reported for more established cryopreservation protocols in other species, but the information gained will provide a basis for further research. Several factors were found to contribute to the survival of sperm during the freezing and thawing process. Egg yolk was found to have a beneficial effect on membrane integrity. This was less pronounced on post-thaw activity. The combination of base medium and cryoprotectant was also important.

The beneficial effects of egg yolk in sperm cryopreservation extenders are well established in mammalian species (Phillips 1939, Watson and Martin 1973) and in some teleost species (Babiak et al. 1999). The components of egg yolk providing protection to sperm during freezing are low-density lipoproteins and phospholipids (Pace and Graham 1974, Watson 1976), although the exact mechanism of action is unknown (Andrabi 2008). It has been suggested that these components help to stabilize the plasma membrane during freezing by binding to sperm (Watson 1975, Quinn et al. 1980), and affect the way sperm respond to osmotic changes in the presence of cryoprotectants by altering membrane permeability (Holt 2000). In the present study, increasing egg yolk concentration resulted in a significant increase in post-thaw membrane

integrity, indicating that egg yolk had a protective effect on the sperm plasma membrane of sparsely spotted stingaree. The effect of egg yolk on post-thaw activity was less clear. Increasing egg yolk concentration led to a general but non-significant increase in initial post-thaw activity, but activity declined within 5 min after thawing in the majority of treatments. It may be that although egg yolk had some protective effect, on its own it was not sufficient to prevent damage caused by other factors present after thawing. Given this, and the complicated procedure required for adding egg yolk to extenders, future work on cryopreservation of elasmobranch sperm should consider the usefulness of egg yolk on a species-by-species basis rather than as an essential additive to sperm extenders.

The less pronounced decline in post-thaw activity observed in extenders using TSK as base medium may be due in part to the cryoprotective effects of sucrose. It has been shown to be a beneficial additive to extenders for bovine (Woelders et al. 1997) and human (Hossain and Osuamkpe 2007) sperm. Sucrose has been shown to stabilize the plasma membrane and reduce membrane permeability during cryopreservation by interacting with membrane phospholipids (Strauss et al. 1986, Anchordoguy et al. 1987) and may also reduce solution effects by increasing the amount of unfrozen water outside the cell and changing the crystallization pattern of the extracellular solution (Woelders et al. 1997, Holt 2000). The sucrose concentration used in the present study (25 mM) was relatively low compared to the total osmolality of the extender (1100 mOsmol/Kg) and further investigations using higher sucrose concentrations are warranted.

Given the decline of elasmobranch species in the wild and their vulnerability to over-exploitation (Fowler et al. 2005), storage of genetic material from these species for potential use in future breeding programs is of great importance. Although artificial breeding of elasmobranch species is not yet an established management technique, early work on model species has been promising (Luer et al. 2007). It is anticipated that sperm cryopreservation will be an essential component of future breeding programs utilizing artificial spawning of elasmobranch species in aquaria to reduce the need for capture of animals from the wild. The results from the present study will provide a basis for continued research on cryopreservation of elasmobranch sperm. Although the extender containing ER with glycerol produced the highest post-thaw membrane integrity and activity, the longer duration of activity observed in extenders containing TSK with glycerol or DMSO makes the latter a better choice for further research. Future studies should investigate the effect of different concentrations of sucrose and cryoprotectants (glycerol and DMSO) across a range of freezing rates, and control sperm concentration to remove variations in the ratio of sperm-to-cryoprotectant as a source potential of error.

Acknowledgments

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New Chapter

Amphibian Conservation and Cryopreservation of Sperm, Cells and Tissues

Robert Browne and Chester R. Figiel Jr.

Introduction

Spallanzani (1789) reported that frog sperm could fertilize eggs after cold storage (Luyet and Gehenio 1940). Rostand (1946) described successful storage of frog sperm for d in glycerol at below-zero temperatures, and Barton and Guttman (1972) examined the effects of several extenders and cryoprotectants on the cryopreservation of sperm from the American toad *Bufo americanus*. Yet it was not until the end of the 20th century, with the realization that amphibian populations were in drastic decline, that cryopreservation was recognised as a practical method for maintaining genetic variation in conservation breeding programs (Browne et al. 1998).

This need created impetus for further research into amphibian cryobiology. It was shown by Mungano et al. (1998) that testicular sperm of wood frog *Rana sylvatica* could retain cell integrity (viability) but not motility after freezing, and that cryopreserved testicular sperm of European common frog *R. temporaria* (Kaurova et al. 1996) and cane toad *Bufo marinus* (Browne et al. 1998) sperm could fertilize oocytes. These methods were used to recover viability from cryopreserved sperm from a range of other anuran species (Browne et al. 2002b), and to fertilize oocytes of African clawed frog *Xenopus laevis* (Mansour et al. 2009) and testicular sperm and hormonally induced sperm of *R. temporaria* (Mansour et al. 2010, Shishova et al. 2011). The early 21st century has seen rapid advances in the cryopreservation and use of amphibian sperm for artificial fertilization, and to a lesser extent somatic cells and tissues. However, of the three orders of amphibians: anurans (frogs and toads), caudata (salamanders), and caecilians (secretive, limbless burrowing animals that resemble snakes or worms), the use of cryopreservation for reproduction has been limited to anurans and caudates. In practice, cryopreserved sperm and somatic cells are used to preserve germ lines and strains of *Xenopus* for biomedical applications (Sargent and Mohun 2005) and nuclear transfer (Dinnyes et al. 2007).

Figure 1. Terrestrial caecilians such as the makudu *Boulengerula uluguruensis* are perhaps the most morphologically and reproductively specialized of amphibians. The mothers of this species feed their young on their skin.

Browne, R. and C. R. Figiel Jr. 2011. Amphibian Conservation and Cryopreservation of Sperm, Cells, and Tissues. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 345-365.

The Amphibian Crisis and Cryopreservation

The extinction crisis in amphibians has evoked an unprecedented response from the world conservation community. Fifty percent of the world's 6000 amphibians are in decline and approximately 500 endangered species require *ex situ* programs for their survival (IUCN 2008). Some of these species are threatened by emerging diseases including chytridiomycosis (Skerratt et al. 2007, Lips et al. 2008), or ranavirus (Robert 2010) which cause declines and extinctions independent of habitat quality. Other threats are habitat destruction through deforestation, agriculture, water extraction, and climate variation (Mendelson 2006, Lips et al. 2008). These threats are unlikely to be ameliorated in the immediate future. To face this challenge, conservation breeding programs (CBPs) are being implemented to perpetuate threatened amphibian species and their genetic variation. Where practicable these programs include *in situ* components such as field studies, habitat restoration, and rehabitation and population supplementation (IUCN 2005). Sustainable population management of many amphibians will also rely on the micro-economic and social benefits to local communities (Silva 1998). These benefits include improved environmental services (Cox 2002) and aquaculture and harvesting of amphibians for consumption or display (Miles et al. 2004).

Figure 2. Genetic studies have recently divided tiger salamanders of the genus *Ambystoma* into several species and many genetic sub-populations. The most promising way to perpetuate their genetic variation is through cryopreserved banks of sperm and somatic nuclei.

Conservation breeding programs must perpetuate the genetic variation of threatened species that defines their niche in ecosystems. However, with many threatened amphibians the loss of genetic variation from small populations is accelerated by habitat fragmentation, meta-populations, and genetic sub-populations (Hendrick and Kalinowski 2000) (Figure 2). Releasing genetically incompetent amphibians may result in lowered resistance to disease, and poor reproduction and adaptability. Therefore, amphibian CBPs must maintain genetic variation of source populations to maximize the potential of rehabitation for supplementation projects (Reed et al. 2007). The need to perpetuate genetic variability supported by gene banking has been recognized in other vertebrates (Holt et al. 2003, Swanson et al. 2007), but except for fish, has rarely been implemented (Baker et al. 2007).

Amphibians have some of the highest levels of genetic variation between adjacent sub-populations known in vertebrates. Their sub-populations have evolved different co-adapted gene complexes, and offspring from among-population crosses may have reduced fitness (Sherman et al. 2008). The fine-scale genetic variation of these species must be maintained to optimize the chances of success in rehabitation programs.

The perpetuation of genetic variation in CBPs, even for only tens of generations, requires the maintenance of hundreds to thousands of animals (Schad 2008). For security these amphibians must be distributed among several institutions. Even then, unpredictable and catastrophic mortality, or the adaptation of captive populations to husbandry conditions, can substantially reduce genetic variation over short periods (Frankham 2008). The cost and management challenges of conservation breeding programs relying on large *ex situ* populations are also formidable (IUCN 2007).

The perpetuation of amphibian genetic diversity through CBPs is major goal of on the International Union for the Conservation of Nature, Species Survival Commission (IUNC 2005), and the World Association of Zoos and Aquariums (Pavajeau et al. 2008). Therefore, CBPs need to adopt and develop genetic resource banking (gene banking) to perpetuate amphibian genetic variation and diversity (Moore and Church 2008).

Amphibian Cryopreservation: Conservation, Biotechnology and Aquaculture

Development in the cryopreservation of amphibian cells and tissues for the perpetuation of genetic variation in CBPs will be fostered by development in concert with other biotechnologies and in aquaculture. A number of amphibian species are already farmed for consumption (e.g., bullfrog *R. catesbeiana*) (Nootprapan and Pariyanonth 1991) and for consumption and medicine (Chinese giant salamander *Andrias davidianus*) (Wang et al. 2004), or for biotechnological applications (*Xenopus*) (Beck and Slack 2001). Conservation breeding programs, amphibian aquaculture, and biotechnological applications each have different emphases in the management of genetic variation. For example, in aquaculture emphasis includes development and perpetuation of traits that optimize growth and survival in culture, and in biotechnology emphasis includes development of novel gene lines (Beck and Slack 2001).

The use of fresh or frozen cells and tissues enables application of a comprehensive suite of conservation tools and strategies. Cryopreservation of viable nuclei in sperm or other cell types can economically and securely retain genetic variation or amphibian genotypes indefinitely (Buchholz et al. 2004). Cryopreserved male and female gametes, or somatic tissue or nuclei, can be used to reduce the number of individuals needed to perpetuate genetic variation in CBPs from hundreds to tens of individuals (Schad 2008). These samples can be used to produce offspring with the full complement of natural genetic variation, or research strains that display specific desirable traits (Sargent and Mohun 2005). Oocytes can be fertilized directly by placing of motile sperm on oocytes, or indirectly through intracytoplasmic sperm injection (ICSI).

Using ICSI or nuclear transfer, these gene banks are capable of generating amphibians from oocytes (Uteshev et al. 2002, Sargent and Mohun 2005). Refrigerated storage of sperm or eggs at above-freezing temperatures also enables artificial fertilization when the production of gametes between preferred pairs is temporally or spatially asynchronous (Browne et al. 2006a,b). Cells and tissues stored in cryobanks (even without the use of cryoprotective protocols) are increasingly used for a variety of research purposes including molecular biology, pathology, toxicology, and environmental research (O'Rourke 2007). Studies with other vertebrates show that sperm are capable of generating new individuals after storage for 1-8 d in -80 ºC freezers (without cryoprotection) (Ogonuki et al. 2006), and that mouse sperm held at high osmolarities at room temperature for 1 wk and then at -20 ºC for as long as 3 months can be fertile. These technologies could enable the use of a new range of stored samples, transport of sperm from the

field without the need for ice, and increase the range of storage facilities from the current reliance on liquid nitrogen to freezers at -80 ºC and -20 ºC.

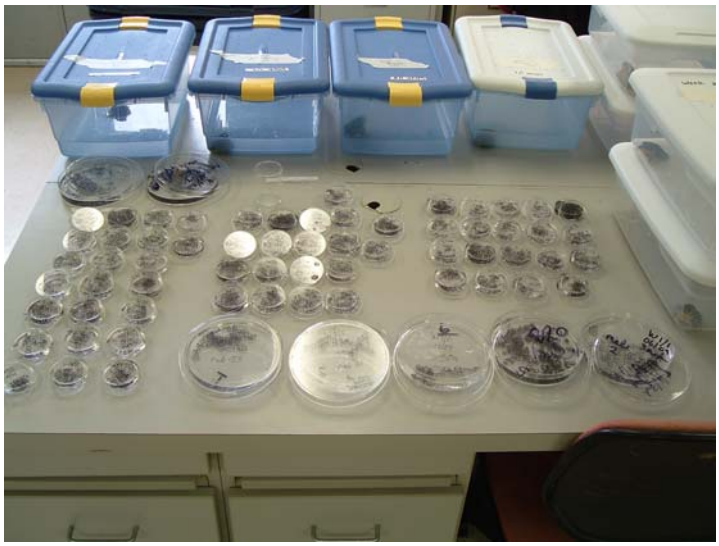

Figure 3. The use of cryopreserved sperm enables perpetuation of genetic variation through use of multiple males to fertilize oocytes of multiple females as shown for a typical experimental fertilization trial.

Fresh or cryopreserved somatic cells can each be used for nuclear transfer or in the development of cell lines. Standard methods for cultivation and cryopreservation of somatic cell lines of *Xenopus* are available, and cell lines have been established for a range of other amphibians (Kumar and Brockes 2007). Simple techniques including sampling of fin clips with fish have provided cell lines with subsequent recovery of viable cells after cryopreservation (Mauger et al. 2006) and may also provide sampling methods for cells from tadpoles.

The use of cryopreserved anuran sperm to achieve artificial fertilization has proven to be more challenging than with many mammals, birds, and fishes. Studies have shown high levels of viability after sperm cryopreservation (Buchholz et al. 2004, Hopkins and Herr 2004) but only recently has a range of techniques been developed that promise general applicability to various amphibian taxa (e.g., Kuo et al 2008, Masour et al. 2009, 2010, Shishova et al. 2011). In contrast, the sperm of more than 200 fish species from a wide range of taxa have been cryopreserved (Figiel and Tiersch 1998, Kopieka et al. 2007, Rana 2007) including the sperm of internally fertilizing species (Huang et al. 2004).

Reproductive Strategies

Amphibians have the widest diversity of reproductive strategies of all tetrapods (see reviews by Salthe 1969, Salthe and Duellman 1973, Duellman and Trueb 1994, Sever 2003). These reproductive strategies have evolved in response to selection pressures (e.g., desiccation, predation, competition, and diseases or parasites), that affect each amphibian life stage: egg, larval, and adult. Amphibian species lay their eggs in burrows, in bromeliad plants, on leaves, in foam nests, within skin pockets in males, in all kinds of water storages, and in every kind of freshwater habitat whether lentic and lotic. Additionally several hundred species reproduce through direct development (elimination of the larval or tadpole stages), an ecological adaptation that has evolved repeatedly and may have been critical to the evolutionary success of several amphibian groups. Further, the transition from aquatic to terrestrial spawning has allowed amphibians to colonize and adapt to more varied environments. There is also an amazing diversity of reproductive strategies of parental investment before and after egg deposition.

Figure 4. Amphibian reproduction in is highly diverse and includes direct development from eggs suspended above ponds on leaves, such as shown here for the red-eyed tree frog *Agalychnis callidryas*. The large size of most amphibian eggs and their external development enables easy injection of viable sperm or nuclei, and the harvesting of totipotent cells from blastocysts.

The wide variation in amphibian reproduction demands a range of protocols for storage and use of sperm or nuclei. Efforts to collect gametes are affected by the physiological response of amphibians to temperature, rainfall, photoperiod, and other environmental cues affecting sexual maturation (Whitaker 2001). Spermatogenesis and oogenesis can be impaired during drier, colder seasons, and natural hormone cycles provide cues to the optimal artificial release of quality gametes. Even with amphibians out of breeding condition, artificial induction of oocytes or sperm through hormone administration can increase reproductive output (Obringer et al. 2000, Whitaker 2001, Michael et al. 2004, Browne et al. 2006b).

Additionally sperm morphology may have evolved in response to different methods of reproduction and can influence cryopreservation success. In part this may be associated with the complex reproductive history of amphibians. Following externally fertilizing fish ancestors, early amphibians were internal fertilizers, then most anurans, and some caudates reverted to external fertilization, with some anurans reverting to internal fertilization (Jamieson 1991). All caecilians have retained internal fertilization (Duellman and Trueb 1994).

Sperm Morphology

The structure of amphibian sperm has exhibited rapid and divergent evolution. Sperm morphology in amphibians has been used to examine phylogenetic relationships (e.g., Lee et al. 1993, Kwon and Lee 1995, Scheltinga and Jamieson 2003, Costa et al. 2004, Scheltinga and Jamieson 2006, Qin et al. 2008) and has been used to complement genetic information for systematic purposes. It has been suggested that the diverse array of sperm morphologies exhibited by amphibians have resulted from the complex adaptation from internal to external to internal fertilization (Lee et al. 1993). This hypothesis suggests that the occurrence of complex sperm in basal anurans, salamanders, and caecilians, and the high diversity of modifications in several derived anuran families, are evidence of an ancestral condition with a complex sperm in amphibians. That is, that there is a general trend in anurans towards the simplification of sperm through the loss of structures due to reversion to external fertilization.

A few studies have examined the significance of variation in amphibian sperm morphology. It was suggested that sperm having longer heads and thus more nuclear material, may be correlated with evolutionary plasticity in plethodontid salamanders (Wortham et al.

1977), and it was also suggested that differences in sperm from *Ambystoma* salamanders may be correlated with the differences in the thickness of oocyte membranes (Brandon et al. 1974). It has been suggested in myobatatrachid frogs that sperm length was correlated with selection pressures related to sperm competition and egg morphology (Byrne et al. 2003). The specific selective pressures on sperm morphology are unclear as sperm structure corresponds with phylogeny more than with fertilization biology in amphibians (van der Horst et al. 1995a,b).

The basic sperm morphology of amphibians consists of a head with acrosome or acrosomal cap, midpiece, and a tail structure. Generally the head is elongated and cylindrical and tapers anterior to a barbed acrosomal cap. The midpiece contains one or two centrioles anterior to the head and at the proximal end of the axial rod. The tail consists of a supporting axial rod attached to a lateral flagellum (monoflagellate spermatozoa) or two flagella (biflagellate sperm) with undulating membranes. Biflagellate sperm have been described in unrelated groups in anurans (Aguiar et al. 2003, Veiga-Menocello et al. 2007) and in the salamander family Sirenidae (Austin and Baker 1964), which suggests that this trait has multiple independent origins. It was put forward that anuran biflagellate sperm resulted from a simplification which included the loss of accessory fibers and the loss of an undulating membrane (Lee et al. 1993). However two complete flagella with undulating membranes and axial fibers were described in dendrobatid frogs (Veiga-Menocello et al. 2007).

Besides the studies listed above, additional studies on comparative sperm morphology show the uniqueness and diversity among amphibians and the reader is referred to these studies for addition information. For example, see Wake (1993) and Scheltinga et al. (2003) for descriptions of sperm morphology of caecilians, and Martan and Wortham (1972) and Wortham et al. (1982) for descriptions of sperm morphology of some salamander species.

Collection of Gametes

Sperm Collection

Spermatozoa can be collected through the excision and maceration of testes, or through hormonal induction and expression in urine. The testes lie attached to the dorsal surface of the kidneys and are elongated to spherical in shape (Byrne et al. 2002). Once removed the testes can be washed in cold simplified amphibian Ringer (SAR) (Browne et al. 1998) to remove blood and other contaminants and placed in tubes on ice slurry. Sperm should be collected from testes as soon as possible by their maceration into cold SAR, or in cryodiluent to form a suspension if the sperm is to be cryopreserved. Sperm suspensions in SAR can be stored for hr or d at near-freezing temperatures and be used for artificial fertilization.

Macerated testes are used to make a concentrated sample of sperm suspension by weighing the testes and placing with an equivalent volume of SAR or cryodiluent (e.g., 1 g to 1 mL). Testis weight, male size, and sperm production are highly correlated (Edwards et al. 2004). The testes are macerated in the solution with forceps, or chopped with a sharp blade to form sperm suspensions thatcontain ionic and organic components derived from the testes. These organic components may assist in cryopreservation by providing a source of lipids, antioxidants and other cryoprotectant compounds. The ionic components provide the osmolarity of blood plasma.

Sperm and spermatophores can be collected through hormonal induction administered through injection. However, until recently sperm in useful quantities was only obtained from a few species, and with some species the small quantity of sperm limited use largely to ICSI. The

effectiveness of hormones has also been shown to vary widely among species. It is possible to induce spermiation and amplexing (breeding) behavior in male amphibians by use of human chorionic gonadotropin (HCG) or luteinizing hormone-releasing hormone analogue (LHRHa). human chorionic gonadotropin (HCG) appears to be the most reliable hormone for the induction of spermiation in some toads (Browne et al. 2006a,b). Spermiation was induced from seven species of anurans with LHRHa (Waggener and Carroll 1998a) and LHRHa was used to induce sperm from toads (Obringer et al. 2000). If LHRHa was administered initially in inadequate doses of ~0.12 µg/g for many species, with recent studies showing doses of ~1.16 µg/g being generally effective (Shishova et al. 2011). Of the different types of LHRHa available, des-Gly^{10}, D-Ala^{6}]-LH-RH ethylamide acetate hydrate (Sigma Chemical Corp., St. Louis, Missouri, USA; L4513) has been found to be generally reliable (Obringer et al. 2000, Wright and Whitaker 2001, Michael et al. 2004, Toro and Michael 2004, Browne et al. 2006a,b). However, a new range of hormones are being tested that appear to induce anuran spermiation (Trudeau et al. 2010, Scilla 2010). Hormonally induced sperm expressed in urine is highly motile and must be held refrigerated to maintain motility.

Topical hormone administration has been proposed as an alternative to intraperitoneal injection for the release of sperm. The ventral dermal application of 100 or 10 µg of LHRHa in 40% dimethyl sulfoxide (DMSO) was shown to induce spermiation in male American toad *B. americanus* and Gulf toad *B. valliceps* (Rowson et al. 2001). This method failed to induce spermiation in *G. rosea* (Aimee Silla, personal communication). Sperm will mainly be expressed between 1 and 12 hr after the administration of hormones (Browne et al. 2006a,b).

With some anurans, notably toads, it is possible to collect sperm by stimulating the animal to urinate. The toad is dried to remove superficial water and held anterior to the pelvic girdle by one thumb and index finger above a 150-mm Petri dish and gently massaged along its anterior surface. This technique usually promotes urination within 60 sec, and the volume of sperm suspension can be several mL.

It is also possible to collect sperm by using a pipette to irrigate the cloaca or by cannulation of the efferent ducts with species that do not spontaneously urinate. This method was used to obtain sperm from eight anuran species including *X. laevis, R. pipiens, L. laevis and L. llanensis* (Waggener and Carroll 1998b). Sperm suspensions obtained from 1-5 hr after injection into the dorsal pelvic area, either by mechanical stimulation or by cloacal lavage varied in volume from 8 µl to 7 ml and the cell densities ranged from 4×10^5 to 4×10^7/ml (Waggener and Carroll 1998b). In some small species, sperm can be sampled by gently inserting the end of a fire-polished microcap into the cloaca (Silla 2010, Silla and Byrne 2010).

Oocyte Collection

Oocytes for *in vitro* fertilization are normally obtained through hormonal induction using HCG or LHRHa, and more recently with anti-dopaminergic drugs (Trudeau et al. 2010). Human chorionic gonadotropin has proven effective in ovulating a variety of anurans including the African clawed frog (Hogben 1939), Wyoming toad *B. baxteri* (Browne et al. 2006b), Tungara frog *Physalaemus pustulosus* (Lynch et al. 2006), and northern leopard frog *R. pipiens* (Bergers and Li 1960). However, there are many species that ovulate poorly with HCG including the Indian skipper frog *R. cyanophlycti* (Ramaswami and Laksham 1958), the Puerto Rican coqui *Eleutherodactylus coqui* (Michael et al. 2004), the cane toad (Browne et al. 1998), and Fowler's toad *B. fowleri* (Browne et al. 2006a). In general hormone treatments within related amphibian species have shown similar responses such as in the *Litoria aurea* group (Browne et al. 2007),

Bufo (Browne et al. 2006a,b), *Xenopus* (Mansour et al. 2009), ranids (Shishova et al. 2011), and *Pseudophryne* species (Silla 2010).

Combinations of HCG with LHRHa have been more effective than either hormone alone (Browne et al. 2006a,b) and with progesterone improved the number and quality of oocytes in Fowler's toad (Browne et al. 2006a). Toads administered 1.18 µg/g of LHRHa with progesterone had superior ovulation compared to 0.61 µg/g of LHRHa alone, or 15 IU HCG/g with 0.12 µg of LHRHa (Browne et al 2006a,b). The use of 10 µg/g of the anti-dopaminergic drug metoclopramide with 0.4 µg/g of LHRHa was shown to produce high levels of amplexing and spawning in a range of species (Trudeau et al. 2010). The use of 2 µg/g LHRHa (Leuprorelin acetate; Lucrin®) with arginine-vasotocin (AVT; arg^8- vasotocin acetate salt) (Sigma-Aldrich Chemical Co.) provided the best induction with Günther's toadlet *Pseudophryne guentheri*.

After ovulation, oocytes can be collected through spawning, stripping, or the excision of oocytes from the ovaries. Oocyte production is highly correlated with female body weight (Edwards et al. 2004). Spawning females should be placed in plastic boxes with a shallow depth of SAR to enable the *in vitro* fertilization of oocytes (Browne et al. 2006a,b). If females ovulate but do not spawn, it may be possible with some species to strip oocytes through palpation (Whitaker 2001). This is successful in some groups including ranids but it is not generally suitable for others including toads where the egg mass is produced as paired strings. The mature oocyte mass may be obtained directly from the oviduct by excision in egg-bound females in which palpation does not release the oocytes (Browne et al. 1998).

Handling and Activation of Sperm

Optimal handling techniques for sperm are important for maintaining motility and viability during short-term storage, cryopreservation and *in vitro* fertilization. Optimization of handling techniques must consider the complex interactions that occur among motility, osmolarity, fertilization, and sperm quality (van der Horst et al. 1995a,b). Amphibian blood plasma osmolarity and that of testicular tissue is ~290 mOsmol/L. Sperm from externally fertilizing amphibians become increasingly motile as the osmolarity declines from 180 to 5 mOsmol/L (pond water) (Hollinger and Corton 1980, van der Horst et al. 1995a,b). However, the maximum percentage, degree, and duration of motility generally occur at osmolarities of 40 mosmol/L or higher (Sargent and Mohun 2005, Browne et al. unpublished). There are fewer studies on the activation of sperm of internally fertilizing species. Nevertheless, high levels of viability and some motility have been recovered from cryopreserved *C. alleganiensis* sperm (National Geographic 2010), and protocols were developed for artificial fertilization with axolotyl sperm (Mansour et al. 2011).

When activating sperm the fraction of the osmolarity in the sperm suspensions produced through non-penetrating cryoprotectants affects sperm motility as the difference between internal and external osmolarity mainly controls the activation of motility. With *B. marinus* suspensions containing 10% (w/v) of the non-penetrating cryoprotectant sucrose (at 290 mOsmol/L) and 10% (v/v) of the penetrating cryoprotectant DMSO when diluted at 2:1 (water:suspension) recovered high motility (Browne et al. 1998). In the same cryodiluent thawed sperm of *X. laevis* showed the highest percentage of motile sperm and velocity with a dilution of 3:1 (Christensen et al. 2004), and the percent motility of *B. marinus* sperm was highest at 6:1 compared to a 20:1 dilution (Fitzsimmons et al. 2007).

The chemical characteristics of the activation solution including pH, tonicity, stimulants, and temperature affect the percentage and velocity of sperm motility and its longevity. The stimulation of sperm motility through optimization of osmolarity and media may improve motility and fertilization rates (Kouba et al. 2003, Mansour et al. 2011). A number of other substances have been tested to stimulate or extend the motility of amphibian sperm including caffeine, and ATP/cyclic AMP, but their effects appear insignificant. Nevertheless, theophylline increased the motility of *B. marinus* sperm (Fitzsimmons et al. 2007). As temperature declines to 0ºC, metabolic and chemical processes minimize motility and metabolism (Browne et al. 1998), and if sperm is to be held for more than a few min it should be placed on ice slurry.

Figure 5. Over the last decade aquaculture of the Chinese giant salamander *Andrias davidianus* has expanded to thousands of farms and production of hundreds of thousands of salamanders. The rapid selection of genetic strains in captive populations is shown by the range of color variations in these 5-yr old animals. They can eventually grow to 2 m and 59 Kg.

Assessment of Sperm Viability

The most commonly used methods for determining the quality of sperm are: percentage and degree of sperm motility (Hollinger and Corton 1980, Browne et al. 1998); sperm plasma membrane integrity (Beesley et al. 1998, Costanzo et al 1998, Mugnano et al. 1998, Buchholz et al. 2004, Michael and Jones 2004, Fitzsimmons et al. 2007), and fertilization rates (Barton and Guttman 1972, Browne et al. 1998). Sperm viability can also be assessed through morphology, mitochondrial function, capacitation and the acrosome reaction, oocyte binding, sperm genetic integrity (Mocé and Graham 2008), and live/dead fluorescence viability kits.

The percentage and degree of motility is normally assessed with three fields under a phase-contrast microscope with 30-80 sperm per field. Wiping with aqueous solutions of 1% bovine serum albumin (BSA) or 1% polyvinyl alcohol can help prevent sperm from adhering to glass slides and cover slips (Chapeau and Gagnon 1987). The effects of BSA as a cryoprotectant may be confounded by its action in preventing adhesion. Sperm suspension is diluted with water to achieve an optimal osmolarity of between 5 and 80 mOsmol/L. The percentage of motile sperm can be measured by two methods: if the flagellum is active (Browne et al. 1998), or through the percentage of sperm moving with velocity (Browne et al. 2006a,b).

Caution must be used when comparing assessments by the two methods because sperm that are not moving can be considered in the percentage motile with the first method but not the second. The degree of movement of sperm is commonly assessed as a scale with “zero” having no forward progression and “five” for sperm with vigorous progressive motility (Browne et al.

1998). Computer-assisted sperm analysis (CASA) can be used to assess anuran and caudate sperm average path velocity, linearity, and wobble, and percent motility (Mansour et al. 2010).

Short-Term Storage of Sperm

The short-term storage of unfrozen sperm, from hr to wk through low temperature or high osmolarity, has been limited to externally fertilizing anurans (Hollinger and Corton 1980, Browne et al. 2001, 2002b,c). In externally fertilizing anurans the storage period of sperm in SAR is a matter of hr unless near-zero temperatures are used, which can prolong storage to weeks. Near-zero temperatures can also extend storage life of otherwise motile sperm at low osmolarities to several d (Browne unpublished). *Bufo marinus* sperm retained greater than 50% motility when stored in SAR for as long as 5 d at 4 ˚C, and motility did not cease until 15 d of storage (Browne et al. 2001). Sperm stored for 6 d has also been shown to recover motility after cryopreservation (Browne et al. 2002b,c). Amphibians that have died and are stored at 4 ºC may provide viable sperm for as long as 24 hr (Browne unpublished).

Sperm storage to maximize motility can be extended by osmolarities high enough to prevent activation. The development of storage media for amphibian sperm has been limited to saline solutions. However, in other vertebrates a variety of storage media are used to extend storage time (e.g., Holt 2003). The addition of a saline solution (20 mM NaCl, 1 mM KCl, 1 mM Mg_2SO_4, 1 mM Ca_2Cl, 3 mM $NaHCO_3$, 10 mM Tris, pH 8.5, osmolarity 65 mOsmol/L) improved fertilization of axolotl sperm (Mansour et al. 2011). When the pH of the fertilization solution was increased to 10 or greater, the hatching rate was significantly increased. The use of fertilization solutions with osmolarities of less than 150 mOsmol/L, decreasing hatch rates (Mansour et al. 2011).

Short-term Storage of Oocytes

The period that amphibian oocytes remain fertile can be lengthened by increasing osmolarity to slow gel hydration, or by lowering temperature to reduce metabolism (Browne et al. 2001). Oocyte fertility was extended by storing in SAR for the spotted marsh frog *Limnodynastis tasmaniensis* (Edwards et al. 2004), *B. marinus* (Browne et al. 2001), *B. fowleri* (Browne et al. 2006a), and *B. Baxteri* (Browne et al. 2006b). DeBoer's solution at three times the standard concentration, extended the fertility of *Xenopus* oocytes (Hollinger and Corton 1980).

The effect of gel hydration on blocking the passage of sperm varies among species. This may be a consequence of the wide variety of gel types found in amphibian spawns. In the spotted marsh frog, oocytes are spawned in a foam mass, and can be stored in 5 mOsmol/L (pond water) for 2 hr without a decline in fertility. In addition, fertility was extended to > 4 hr in osmolarities of 124-271 mOsmol/L (Edwards et al. 2004). In contrast, after hydrating for 1 hr, the oocyte gel coat in the Japanese newt *Cynops pyrrhogaster* prevented fertilization. The fertilization period was increased through storage in De Boer's solution or Holtfreter's balanced salt solution (Matsuda and Onitake 1984). The problems with gel hydration can be prevented by removing gel from oocytes. Besides the effect of egg gel, fertilization and development rates tend to decline as the storage period of oocytes increases. Delayed fertilization of *Xenopus* oocytes resulted in abnormal development during gastrulation, neurulation, and organogenesis. (Wakahara et al. 2005).

Cryopreservation of Sperm

The development of optimal protocols for the cryopreservation of amphibian sperm have been limited by the small taxonomic range of the few species studied. Nevertheless, the cryopreservation of testicular sperm has achieved successful recovery of viability in a variety of species: *B. marinus* (Browne et al. 1998, 2002a,c), wood frog (Mugnano et al. 1998), Puerto Rican frog (Michael and Jones 2004), and northern leopard frog (Hopkins and Herr 2004). The use of cryopreserved testicular sperm to perpetuate strains of *Xenopus* has been established (Buchholz et al. 2004). In addition, viability was recovered for cryopreserved sperm obtained non-invasively by hormonal induction of *R. pipiens* (Hopkins and Herr 2004).

There are three main factors affecting the success of amphibian sperm cryopreservation: 1) cryodiluents, 2) sampling and acclimation of sperm to cryoprotectants, and 3) freezing rates. Cryodiluents for amphibian sperm generally include non-penetrating and penetrating cryoprotectants. Non-penetrating cryoprotectants include sucrose (~10% w/v) and egg yolk (5-10% v/v), and penetrating cryoprotectants include glycerol, DMSO, and methanol (5-10% v/v) in the final sperm suspension (Browne et al. 1998, Costanzo et al. 1998, Mugnano et al. 1998, Browne et al. 2002a,d). Success was reported using sucrose and egg yolk alone as a cryodiluent for testicular sperm from *X. laevis* and *X. tropicalis* (Sargent and Mohun 2005).

Overall, DMSO has been the preferred cryoprotectant for amphibian sperm. However, a mixture of DMSO and glycerol may prove beneficial in future studies (Beesley et al. 1998). Recent studies have generally used DMSO, sucrose and ionic solutions, sometimes with the addition of egg yolk (Hopkins and Herr 2004). However, dimethyl formamide (DMF) in cryodiluent was superior to DMSO in producing viable larvae from the cryopreserved sperm of *R. temporaria* (Shishova et al. 2011).

Freezing Protocols

The type and complexity of optimal freezing protocols for amphibian sperm varies with the source of sperm, species, and cryodiluents. Freezing protocols have ranged from the freezing of pellets on dry ice (Hopkins and Herr 2004), complex freezing ramps (Browne et al. 1998), ethanol/dry ice slurry (Costanzo et al. 1998, Mugnano et al. 1998, Sargent and Mohun 2005), and recently the use of simple foam freezing boxes to achieve slow cooling (Mansour et al. 2009, 2010, Shishova et al. 2011, Kuo et al. 2008). Acclimation is important to enable the penetration of cryoprotectants into sperm and is accomplished by placing the suspension in a refrigerator for 10 min before cryopreservation. Freezing for 5-7 min at between 5 and 7 °C/min in the vapor at 10 cm above the surface of liquid nitrogen on a polystyrene food tray in an insulated freezing box offered the highest recovery rates for anuran sperm (Shishova et al. 2011, Mansour et al. 2009, 2010). In a limited number of species, different thawing rates have not affected the recovery of cryopreserved amphibian sperm (Browne et al 1998, Hopkins and Herr 2004, Sargent and Mohun 2005). However, the first cryopreservation of hormonally induced sperm used a rapid warming of straws with the suspension placed on a glass hour glass on ice (Shishova et al. 2011). The suspension was osmotically equilibrated and activated in stages.

Sampling and Cryopreservation of Somatic Cells and Tissues

The sampling of somatic cells or tissues of threatened species in CBPs are subject to ethical and logistical constraints. Adult amphibians in the field cannot be euthanized therefore sampling is restricted to the collection of tissue samples or gametes without harming the animal. A possible sampling technique for tissues is fin clips from tadpoles (which often have pieces of tail bitten off in nature) that they quickly regrow. In zoo programs, collection of tissue from living amphibians may be restricted to methods such as hormonal induction of sperm and sampling of embryos before neurulation (BIAZA 2000). Because of biological, demographic, and ethical considerations, sampling of somatic tissue from early growth stages offers the best opportunity to sample pluripotent or totipotent cells. Late larvae and skin samples have yielded cells enabling production of amphibians by nuclear transfer that survive to adults. The range of tissues and species that have provided cell lines are shown in Table 1. However, we know of no instances of cell lines or their cryopreservation from caecilians.

Table 1. Amphibian orders, species, and tissues that have provided cell lines.

Order	Species	Tissue	Reference
Anurans	*Bombina orientalis*	stage 20 embryos	Ellinger et al. 2007
	B. marinus	bladder	Dunand et al. 1985
	X. laevis	kidney	
	R. catesbeiana	tongue	Wolf and Quimby 1964
	X. laevis	endothelial tail cells,	Mawaribuchi et al. 2008
Caudates	*Notophthalmus viridescens*	dorsal iris	Reese et al. 2006
	N. viridescens	limb, , heart, liver,	Ferretti and Brockes 2005
	Pleurodeles waltl	spinal cord	Benraiss et al. 1998
	Ambystoma mexicanum	myofibers	Kumar and Brockes 2007
	A. mexicanum	posterior mesoderm of late gastrulae	Bachvarova et al. 2004

Sampling of Cells from Blastomeres

Blastomeres or early embryos are convenient sources of quality undifferentiated and axenic cells for cell lines or for nuclear transfer. The blastodermal cell mass is sampled by removal of the gel layer surrounding the blastoderm by splitting the gel layer. Once the blastoderm is exposed, the egg yolk can be removed by gentle washing with phosphate buffered saline.

Sampling of Cells from Larvae and Adults

The use of tissue clips from tadpoles can provide a convenient method to sample somatic cells from *in situ* and *ex situ* populations. The single most important factor in collecting a biopsy sample is proper cleaning of the sample site. Protocols are as follows: cleanse the biopsy area using 70% alcohol-drenched gauze. Prepare the recipient vials containing tissue culture medium (minimal essential medium with 10% fetal bovine serum, plus 1% glutamine/penicillin-streptomycin, plus 1% fungizone). Using sterile forceps and scalpel a 5-mm piece of tissue is collected and placed into the vial and capped tightly. The bottle is labeled with species, identification number, sex, institution, and date. The samples can be held (or shipped immediately) unfrozen at low temperatures (4 °C) (Ryder personal communication).

Protocols for Cryopreserving Somatic Cells

Cryopreserved somatic cells of amphibians can provide nuclei for transfer to other cells or oocytes to perpetuate genetic variation (Strüssmann et al. 1999). A suspension with 10% DMSO and 10% sucrose was used for cryopreservation of cells from *R. temporaria* and *B. bufo* embryos for nuclear transfer (Uteshev et al. 2002). The following are websites describe techniques for *Xenopus* cell culture, cell freezing and cloning:

www.chuq.qc.ca/labomoss/Protocols/Protocol%20X%20TC.html
www.xenbase.org/other/methods.do?
tropicalis.berkeley.edu/home/index.html

Intracytoplasmic Sperm Injection

High fertilization rates are essential for the production of large numbers of juveniles. However, the high concentrations of amphibian sperm needed to achieve high fertilization rates with artificial fertilization are unlikely to be sampled from some amphibian species through hormonal induction (Lipke et al. 2009). Even when high concentrations of sperm are sampled, cryopreservation protocols are only developed for a limited range of species. Intracytoplasmic sperm injection using viable but immotile sperm may be the preferred option in these circumstances (Wakayama and Yanagimachi 1998a,b, Kishikawa et al. 1999, Christensen et al. 2004). Even in cases where sperm cryopreservation proves difficult, and with immotile sperm, ICSI could be successful in achieving fertilization (Poleo et al. 2005a,b). *Xenopus laevis* has been widely used for molecular, cellular, and developmental studies and ICSI has been shown to be a straightforward, inexpensive means to reconstitute transgenic frog lines (Buchholz et al 2004). These are two websites describing amphibian ICSI:

www.chuq.qc.ca/labomoss/Protocols/Protocols%20X%20ooc%20emb.html
tropicalis.berkeley.edu/home/manipulate_embryos/microinjection/microinjection.html

Conclusions

There is no single answer for preventing the extinction of amphibian species. However, a multidisciplinary approach that includes captive propagation can provide a way forward in slowing or reversing the losses by producing animals for reintroduction into areas where they have been extirpated, or by maintaining genetically healthy refugia populations to reduce extinction risk. Cryopreservation of gametes, tissues, and cells can assist in these management and recovery efforts and can be an essential and integral part of our mission to stem the loss of amphibians worldwide.

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Cryopreservation of Sturgeon and Paddlefish Sperm

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Introduction

Sturgeons and paddlefish belong to the infraclass Chondrostei, one of the most primitive groups of Osteichthyes. There are only about 25 sturgeon species and two paddlefish species in the order of Acipenseriformes that exist in the world (Rochard et al. 1991, Birstein 1993, Birstein and Bemis 1997). For centuries, these fishes have been highly valued for their role as commercial caviar and for their mild tasting, boneless meat. Today, some of these fishes remain a valuable natural resource; unfortunately, wild populations have declined due to pollution, habitat destruction and in some cases overexploitation for the international caviar trade. Development of induced spawning and husbandry techniques have permitted restoration programs for sturgeons and paddlefish as well as the establishment of farming some of these fishes for food (Mims et al. 2005). Therefore, sperm cryopreservation protocols need to be further developed and optimized so they can provide viable sperm for restoration, conservation of threatened and endangered chondrostei, hybridization, sex manipulation (i.e. gynogenesis and androgenesis) and development of genetically superior broodstock. This chapter will provide information on the chondrostean spermatozoa and a review of past and current cryopreservation protocols.

Morphology of Chondrostean Sperm and Milt Characteristics

Chondrostei spermatozoa are similar to those of other primitive fishes (Afzelius 1978). In general, the spermatozoon has a long cylindrical head capped with a functioning acrosome, a short-mid-piece with several mitochondria and a flagellum with fin-like extensions on both sides of flagellum (Figure 1) (Ginzburg 1972, Brown and Mims 1995, Horvath et al. 2009). The shape of the head is conducive to penetration through one of several narrow micropyle canals in the dense, thick egg membrane (Ginzburg 1972, Linhart and Kudo 1997). The head contains the nucleus and an apical acrosmome that is shaped like a cap with a rounded top. The mid-piece contains the mitochondria and is connected to a sheath surrounding the proximal portion of the flagellum with opposite lateral fins. The presence of a functional acrosome is an important consideration in the freezing and thawing of chondrostean sperm, because a damaged acrosome (i.e. premature acrosomal reaction) causes the sperm not to be viable (Dan 1956, Cieresko et al. 1996a, 1996b, 2000, Brown and Mims 1999).

Chondrostrean males can release large volumes of milt with average concentration of sperm up to 1-1.5 billion spermatozoa per ml. Milt is considered dilute compared to milt of teleost (Linhart et al. 2000, Mims et al. 2000, Piros et al 2002).

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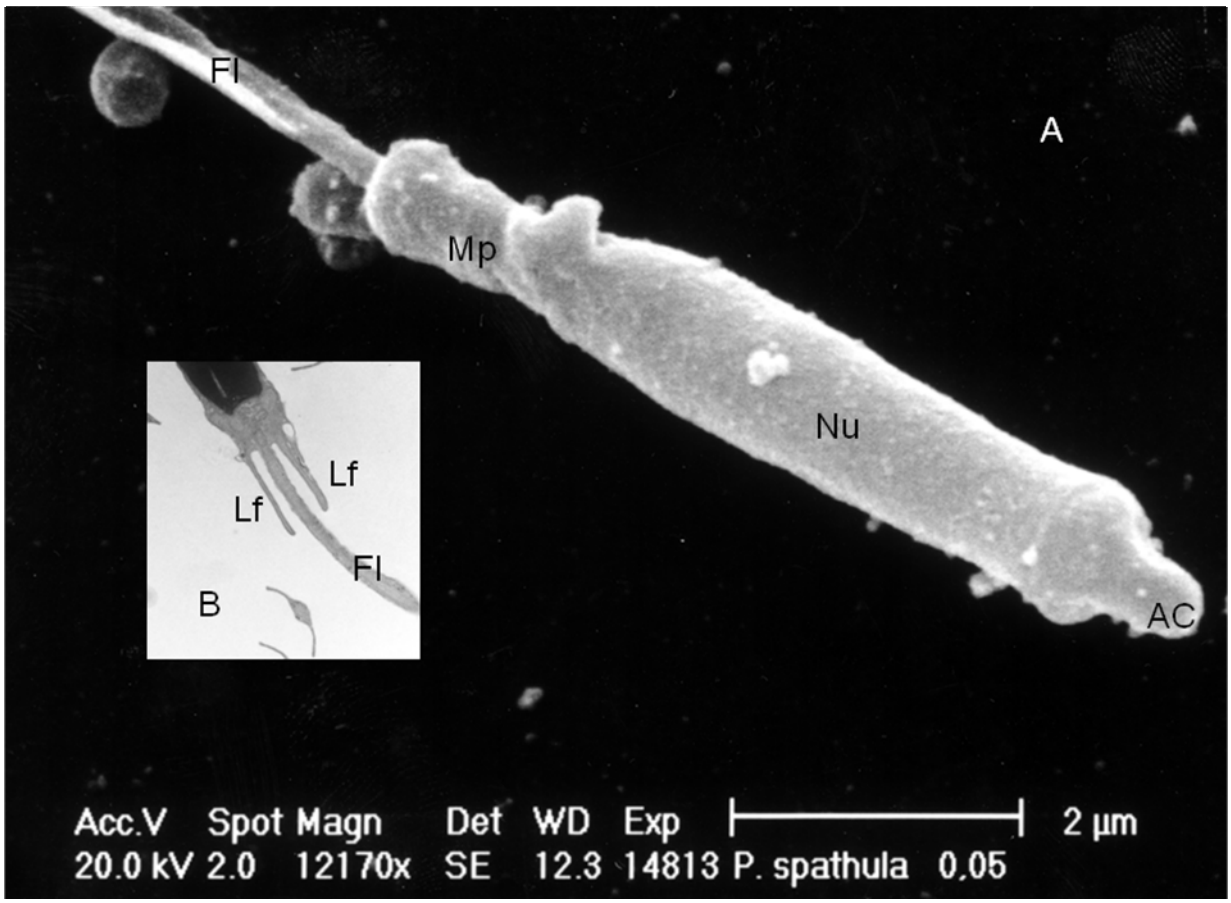

Figure 1. (A) Scan electron micrograph of a paddlefish *Polyodon spathula* spermatozoon (bar=2µm) acrosome (Ac), nucleus (Nu), midpiece (Mp) and flagellum (Fl), (B) Micrograph illustrates the two lateral fins on opposite sides of the flagellum.

Cryopreservation Protocols for Sturgeon Sperm

This section is an updated historical review of the literature as it pertained to research on cryopreservation of sturgeon sperm in the 20th and 21st centuries. During the 20th century, most of the initial research on sperm cryopreservation was done entirely on European sturgeon species, and primarily, if not exclusively, in the former Soviet Union. Published information on sturgeon sperm cryopreservation techniques during this time period was limited but has been reported in reviews by Kopeika and Novikov (1983), Dettlaff et al. (1993) Mims et al. (2000) and Billard et al. (2004).

Burtsev and Serebryakova (1969) first attempted sperm cryoperservation of three sturgeon species: beluga *Huso huso,* kaluga *Huso dauricus* and sterlet *Acipense*r *ruthenus.* Milt was collected and cryopreserved in 5% glycerol in a medium containing 7% saccharose or lactose solution with chicken egg yolk. Also, milt of bester (beluga x sterlet) was cryopreserved using 14% glycerol in a 0.4% NaCl solution. The cryoprotectant medium and milt mixture was frozen in granulated dry ice and stored in liquid nitrogen (LN_2; quick-freeze method). After 15 to 34 d of storage, the samples were thawed in a saline solution and were activated by Ringer's

solution, 0.4% NaCl solution or seawater. The motilities of post-thawed beluga sperm were 50 to 80%, kaluga and sterlet sperm were 10%, and bester (beluga x sterlet) sperm were 40%. However, fertilization rates of samples used to fertilize sturgeon eggs of the same species did not exceed 1%.

Kasimov et al. (1974) froze and thawed sperm of stellate (sevruga) *Acipenser stellatus.* They observed 40% motility of post-thawed sperm and obtained 35% fertilization. Their cryoprotectant medium contained 10% glycerol in a medium of 0.003% chloral hydrate, 0.03% urea and 10% egg yolk. The milt was diluted 1:1 with the cryoprotectant medium, poured into 5 mL centrifuge tubes with caps, frozen in LN_2 vapor to -55 ºC for 2 hr and thawed in a water bath at 40 ºC.

Pushkar et al. (1979) investigated the influence of dimethyl sulfoxide (DMSO), ethylene glycol and glycerol as cryoprotectants and the speed of freezing on sperm cryopreservation for stellate and Russian sturgeon *Acipenser guldenstadti.* DMSO was found to be the best cryoprotectant for sturgeon sperm and when used as a diluent with tris-buffer and egg yolk, 50 to 60% of the post-thawed sperm maintained forward motility. Post-thawed sperm of stellate sturgeon were frozen at -196 ºC and stored for 7 to 23 d, fertilized 64% of eggs, as compared with 77% of eggs fertilized with fresh sperm (control).

In later studies, Pushkar et al. (1980a) reported testing of cryoprotectant medium containing 25% DMSO in 0.2 M (32.5 %) tris-HCl-buffer, 0.1 M (32.5%) HCl, and 10% of egg yolk. The milt was diluted 1:1 in the cryoprotectant medium. The mixture was poured into 0.8 mL ampules and cooled with LN_2 to -12 to -14 ºC at a rate of -0.5 to -5 ºC/min and frozen to -196 ºC. The sperm were thawed in a water bath at 40 ºC; sperm motility was 60%. Pushkar et al. (1980b) used cryoprotectant medium containing 18 to 25% DMSO in 0.1M (65 to 75%) tris-HCI-buffer, 7 to 10% egg yolk and 0.004 to 0.008% of silver iodide. They concluded: 1) tris-HCI-buffer solution was better than Ringer's or Ott-Horton's solution for deactivation and survival of sperm; 2) egg yolk was important to increase the activity and fertilization rate of sperm; and 3) silver iodide decreased the ice crystallization during freezing thereby decreasing the number of damaged sperm. Thus, the following procedure was recommended. Chilled milt (5 ºC) should be mixed continually into the chilled cryoprotectant medium in a 1:1 dilution ratio. The mixture should be allowed to equilibrate for 40 min at 5 ºC. Ampules should be cooled at a rate of 3 to 12 ºC/min by lowering into LN_2 vapor until the samples reach -35 to -45 ºC; thereafter, the ampules should be cooled to the final temperature of -196 ºC at a rate of -130 to -150 ºC/min.

Drokin et al. (1991, 1993) reported motility of 20% for post-thawed sperm of sakhalin sturgeon *Acipenser mikadoi* after cryopreservation with 12% DMSO and 12% yolk in 20 mM tris-HCI buffer (pH 7.5 or 8.0). The milt was chilled to 5 ºC and diluted 1:1 in a chilled cryoprotectant medium. The mixture was allowed to equilibrate for 90 min in 1-mL plastic ampules. The samples were frozen in LN_2 vapor at a rate of 5 ºC/min to -15ºC and at -25 ºC/min to -70 ºC; thereafter, the samples were submerged into LN_2. The samples were thawed in a water bath at 40 ºC for 30 sec.

Cherepanov et al. (1993) reported long-term storage of cryopreserved sperm of sturgeons including beluga, sterlet, Russian sturgeon, bester and ship *Acipenser nudiventris* (Aral Sea origin) from Don River basin. The composition of the cryoprotectant medium and the optimum freeze-thaw regime were similar to the protocols of Pushkar et al (1980b) and Drokin et al. (1991, 1993). Sperm motility was activated by KCl in a tris-HCl buffer. Motility ranged from 10 to 90% after thawing. Samples of frozen sperm from these different sturgeons were stored at the

Low-Temperature Bank of the Institute for Problems of Cryobiology and Cryomedicine of the Ukrainian Academy of Sciences. The motility of the post-thawed sperm after two yr in storage was similar to fresh samples prior to freezing and storage.

Tsvetkova et al. (1996) reported successful fertilization using cryopreserved milt from Siberian sturgeon (*Acipenser baeri*) and sterlet. The milt was diluted in a 1:1 ratio with a cryoprotective medium containing 15% DMSO, 23.4 mM saccharose, 118 mM tris-HCl) pH 8.0 and 20% yolk. The mixture in the tubes was frozen immediately in LN_2 vapor. After thawing for 25 sec at 40 ºC and activating with 50 mM solution of tris-HCI (pH8.0), the sperm motility for Siberian sturgeon was 23 ± 9% compared to 88 ± 4% for fresh sperm (control). For sterlet sperm, post-thawed motility was 15 ± 11% and fresh sperm motility was 68 ± 19%. The fertilization rates using post-thawed cryopreserved sperm were 53 ± 8% (control 89 ± 8%) for Siberian sturgeon and 23 ±11% (control 53 ± 9%) for sterlet.

Andreev et al (1996) determined that 12% DMSO as a cryoprotectant and a slow, three-stage freezing regime provided best results for cryopresrvation of beluga sperm. They found the addition of antifreeze proteins to the medium and quick freezing were not effective. Post-thawed sperm fertilized 43% of beluga eggs compared to 68% fertilization by fresh sperm.

Cierezko et al. (1996) reported the use of 10% DMSO and 0.6M sucrose at a dilution ratio of 1:3 (v:v) for the cryopreservation of lake sturgeon sperm (*Acipenser fulvescens*). Sperm were equilibrated for 1 min and frozen as 0.1-ml pellets on dry ice. After 3 d of storage in liquid nitrogen, the samples were thawed in an activating solution of 20 mM Tris, 30 mM glycine buffer, and 10 mM NaCl and analyzed using computer-assisted sperm analysis. Cryopreservation reduced motility by 70% (fresh - 46%, post-thawed - 14%). Motility characteristics of post-thawed sperm were similar to fresh sperm immediately after activation, but showed decreased straight line velocity and linearity after 5 min compared to fresh sperm.

In 1999, the Third International Symposium on Sturgeon published abstracts that provided information on efforts to develop cryopreservation techniques for sturgeon sperm at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Cherepanov and Kopeika (1999) summarized cryopreservation of sperm from 7 species of sturgeon. The best protocol developed was 10 - 15% DMSO, 100-150 mM Tris-HCl at pH 8.1, and 10 - 15% chicken egg yolk. Sperm were frozen in LN_2 using a three-step process in 0.5- to1.5-mL polypropylene ampoules. No information was given regarding post-thaw motility or fertilization rates. Drokin and Kopeika (1999) reported on the cryopreservation of Siberian sturgeon, Sakhalin sturgeon, and stellate. The sperm of each species were cryopreserved by diluting 1:1 in a 20 mM Tris-HCl, pH 8.0, 15% DMSO, 18% yolk solution, pouring into plastic ampoules and freezing in a three-step program. Ampoules were thawed at 40 ^{o}C for 30 sec. Siberian sturgeon sperm had post-thaw motility of 30 - 40%. Sakhalin sperm sturgeon had 15-20%, and stellate sperm had 30 - 40%. The authors speculated that higher phospholipase activity in the Sakhalin sturgeon sperm was causing damage to the acrosomes, and thereby causing reduced post-thaw motility. Dzuba et al. (1999) reported motility of post-thaw sperm from five species of sturgeon that have been stored for six yr. Post-thaw motility ranged from 20-40% in Russian sturgeon to 60% in ship sturgeon.

Jähnichen et al. (1999) reported a protocol on the cryopreservation of sterlet sperm. Ethylene glycol (12.5% or 17.5% final concentration) was added to an extender composed of 25 mM NaCl, 10 mM Tris, pH 8.5. Sperm were diluted 1:1 with the mixture and loaded into 0.25-mL straws. Samples were frozen in a programmable freezer using two cooling profiles: Profile I. 2 °C to -7 °C: -5 °C/min; -7 °C to -30 °C: -3 °C/min; -30 to -80 °C: -2 °C/min; and, Profile II. 2

°C to -7 °C: -0.5 °C/min; -7 °C to -30 °C: -1 °C/min; -30 to -80 °C: -2 °C/min. Sperm were stored in LN_2 for 1 d or 355 d. Samples were thawed in a 40 °C water bath for either 3 or 5 sec. Highest post-thaw motility was 28 ± 7% using computer-assisted sperm analysis (CASA) and hatch rates were as high as 91%.

Progress in sturgeon sperm cryopreservation has been significant in the 21st century. Sturgeon sperm cryopreservation research has expanded to the rest of Europe, the United States, the Middle East, and East Asia.

Horváth and Urbányi (2000) were the first to report the use of methanol as a suitable cryoprotectant for sturgeon sperm. Sperm of sterlet were diluted at a 1:1 ratio in three basic extenders [sucrose, NaCl and a combination (details were not reported)]. Three cryoprotectants (DMSO, dimethyl-acetamide (DMA) and methanol) were tested at various concentrations. Highest post-thaw motility (46 ± 23%) was reported using the sucrose extender and 10% methanol. The use of methanol also yielded the best fertilization results (22 ± 16% vs. 28 ± 16% in the control); whereas, only 2 ± 4% fertilization was reported for DMSO and none for DMA.

Glogowski et al. 2002 provided a more detailed report on the use of methanol as a cryoprotectant for the cryopreservation of Siberian sturgeon sperm. Three extenders were tested in combination with 10% methanol: 30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl (pH 8.0) modified Tsvetkova's (mT) extender, Jähnichen's extender (Jähnichen et al. 1999) and 20 mM Tris, 400 mM sucrose (pH 8.0) extender. Sperm were diluted at a 1:1 ratio with the freezing diluents containing one of the three extenders and 10% methanol and loaded into 0.25-mL straws. Freezing was conducted in a programmable freezer using a cooling rate of -3.5 °C/min from 4 °C to -15 °C with seeding induced at -7 °C. Straws then were removed from the freezer and placed between two blocks of dry ice for 5 min, then stored in liquid nitrogen. A hatch rate of 30 ± 5% was observed using the Tris-sucrose-KCl extender and was not significantly different from the hatch rate observed with Jähnichen's extender (18 ± 2%) or the control (22 ± 6%).

Urbányi et al. (2004) demonstrated that post-thawed sperm of Siberian, Russian and European sturgeon (*Acipenser sturio*) could successfully be used to fertilize sterlet eggs (3000 eggs per batch) and produced hybridization of three Acipenser species. Post-thawed sterlet sperm were used also to fertilize sterlet eggs (3000 eggs per batch) as a control. The sperm were diluted 1:1 in a diluent containing both mT extender and 10% methanol (cryoprotectant) as reported by Glogowski et al. (2002). Sperm were frozen in 0.5-mL straws placed on 3-cm high Styrofoam frame which was in turn placed onto the surface of liquid nitrogen. Freezing time was 3 min. Hatch rates were 31% for sterlet sperm (control), 50% for Siberian sturgeon sperm, 17% for Russian sturgeon sperm, and 34% for European sturgeon sperm.

Lahnsteiner et al. (2004) evaluated multiple factors for their effects on equilibration motility, post-thaw motility, acrosome reactions, and fertilizing ability of sterlet sperm. The addition of 10% DMSO, 10% methanol, 0.5% sucrose or 0.5% lactose to an extender consisting of 50 mM/L NaCl, 5 mM/L KCl, and 10 mM/L Tris had no effect on equilibration motility after 5 min of exposure. The addition of 0.5% glycine decreased sperm motility. Freezing was found to be optimal at 3-5 cm above the surface of liquid nitrogen. Thawing was optimal at 25 °C for 30 sec. Ten percent DMSO was found to give the highest post-thaw motility rates, followed by 7.5 - 10% methanol, and 7.5% DMSO. Post-thaw motility was decreased by sucrose, lactose, and propandiole. Propandiole also increased acrosome reactions, whereas all other combinations had no effect. Factors for evaluation in the fertilization trials were selected based on the results from the previous experiments. DMSO (10%) and methanol (7.5%) were selected as cryoprotectants

and the addition of 2 mM/L or 5 mM/L KCl to the extender was also examined. Best fertilizations rates were obtained using 7.5% methanol and 5 mM/L KCl.

Tsvetkova et al. (2004, 2006) reported a basic cryoprotective medium consisting of 0.1% of sucrose, 0.08% KCl and 8% methanol for five different sturgeon species (beluga, Russian sturgeon, Siberian sturgeon, sevruga and sterlet) that was developed at the Laboratory of Cryobiology of Institute of Freshwater Fisheries, Russia. The composition of the medium had additional components such as amino acids, sugars, antioxidants or antifreeze glucoproteins for improving the freezing success. Fertilization rates of fresh sperm compared to post-thawed sperm combined in the basic cryoprotectant medium with these additives are indicated in Table 1.

Table 1. Fertilization rates of cryopreserved sperm of five sturgeon species.

Fish Species	Fertilization rate, %	
	Fresh sperm	Post-thawed sperm
Siberian sturgeon	62-88	52-81
Russian sturgeon	35-85	62-78
Beluga (giant sturgeon)	70-98	49-67
Sterlet	79-95	40-62
Sevruga (stellate)	81-93	73-83

Melehova et al. (2004) have developed a patented method for evaluating the rate of sperm damage based on intensity of free-radical reactions. This method permits a more efficient storage of higher quality frozen sperm at the Laboratory of Cryobiology of Institute of Freshwater Fisheries, Russia.

Horváth et al. (2005) reported comparisons of several cryoprotectants and extenders on the sperm of shortnose sturgeon (*A. brevirostrum*) and pallid sturgeon (*Scaphirhynchus albus*). Two experiments were conducted on the shortnose sturgeon and one on pallid sturgeon. The first experiment with shortnose sturgeon sperm tested the modified Tsvetkova's (mT) extender in combination with DMSO or methanol at concentrations of 5, 10 or 15%. The sec experiment tested three extenders mT extender, original Tsvetkova's extender [(oT), Tsvetkova et al. 1996] and modified Hanks' balanced salt solution [(mHBSS), diluted from a commercially available concentrate to an osmolality of 100 mosM/ Kg] and three concentrations of methanol (5, 10 and 15%). The experiment with pallid sturgeon sperm tested mT and mHBSS extenders in combination with 5 or 10% methanol. Sperm samples were frozen according to the protocol described above (Urbányi et al. 2004). In the first experiment with sperm from shortnose sturgeon, the use of 5% DMSO resulted in the highest post-thaw motility (26 ± 13%) however, the highest fertilization (40 ± 15%) and hatch (32 ± 12%) percentages were observed using 5% methanol. In the sec experiment, the highest post-thaw motility (18 ± 10%), fertilization (18 ± 11%) and hatch (17 ± 12%) percentages were found with mT extender in combination with 5% methanol.

In general, the use of DMSO in the first experiment or oT extender in the sec experiment resulted in higher or similar post-thaw motility to methanol or mT extender, however, fertilization and subsequently hatch percentages were significantly lower when DMSO or oT extender were used. Both components are known to have a higher osmolality than that of sturgeon milt plasma, thus, authors speculated that maintaining iso-osmotic conditions is

important for successful fertilization with sturgeon sperm. Extender mHBSS with its osmolality close to that of the seminal plasma yielded intermediate results. This was later confirmed in the experiment on pallid sturgeon sperm when the mT extender resulted in higher post-thaw motility (70 ± 10% with 10% methanol) and fertilization (88 ± 6% with 5% methanol) percentages than that with mHBSS, however, there were no significant differences.

Ciereszko et al. (2006) used a similar protocol on lake sturgeon sperm however, the KCl concentration was increased to 5 mM in the mT extender combined with 10% methanol and stored in 0.25-mL straws. Freezing was conducted on a 3-cm platform floating on the surface of LN_2 for 5 min. Samples were thawed in a 40 °C water bath for 6 sec. Post-thaw motility of cryopreserved sperm was 19 ± 18% with high variability among individual samples. The percentage of pre-hatch embryos was 1.8 ± 0.7% (with 14.3 ± 2.7% in the control). This low hatch rate with cryopreserved sperm as well as in the control indicates that further studies should be conducted and individual species might require slight adjustments of the protocol. In this study, the comet assay was used to determine the level of damage to sperm DNA. Although DNA damage was higher in cryopreserved than in fresh sperm, there was no significant difference.

Liu et al. (2006) used DMSO (12%) as the cryoprotectant for cryopreservation of Chinese sturgeon (*A. sinensis*) sperm. Sperm were diluted 1:3 (v:v) in a diluent containing 8.85 g/L NaCl, 0.20 g/L KCl, 0.40 g/L $NaHCO_3$, and 12% DMSO, and 2-ml of diluted sperm were frozen in 2.5-mL plastic vials. Equilibration times of 0.5-2 hr were determined to be best. A freezing rate of -2 ^oC/min to a temperature of -6 ^oC and holding for 10 min produced the highest post-thaw motility (70%). Samples were thawed at 32 ^oC for about 2 min and two vials were used to fertilize ~200 eggs. Fertilization rate was 84% and hatch rate was 68%.

Mirzoyan et al. (2006) reported positive effects of ascorbic acid and lysine added to the cryo-diluent used in freezing Russian sturgeon sperm. These substances are known to suppress the activity of oxygen-free radicals thus; their addition to the cryoprotective media was considered to improve the survival of cells. Sperm were cryopreserved using an extender that contained a Tris-HCl buffer, egg yolk, sucrose (concentrations of chemicals were not reported) and 15% DMSO with or without 0.01 M ascorbic acid or 0.05 M lysine. Samples were frozen in 2-ml test tubes using a three-step cooling protocol: 5 °C to -5 °C, -5 °C to -70 °C in the vapor of LN_2 (cooling rate was not reported), and -70 °C to -196 °C by plunging into LN_2. Addition of lysine or ascorbic acid improved post-thaw motility and fertilization results, although actual data were not reported.

Grunina et al. (2006) reported the induction of dispermic androgenesis using cryopreserved sperm in stellate sturgeon. For genetic inactivation of maternal chromosomes eggs were irradiated with X-rays; fusion of male pronuclei were stimulated by heat shock. Portions of irradiated eggs were inseminated with cryopreserved and fresh sperm. Sperm samples were frozen in media containing 10% methanol, 7% sucrose and Tris-HCl (pH 7) in 1.5-ml tubes and thawed at 40 °C for 1 min. Hatching rate of 7 and 17% and one-mo survival of 58 and 68% of diploid androgenic larvae were observed after using cryopreserved and fresh sperm, respectively. Combined approach consisting of the dispermic androgenesis and sperm cryopreservation can be used for restoration of endangered sturgeon species.

In 1988, the Low-Temperature Gene Bank of Fish Sperm (i.e. Bank of Cryopreserved Sperm) was founded at the All-Russian Research Institute of Freshwater Fisheries (Rybnoe, Moscow Province, Russia). One of the main objectives of the Gene Bank was to preserve the genetic diversity (gene pool) of sturgeons. In August 2008, the total volume of approximately

400 sperm samples from different sturgeon species was about 8000 mL (Tsvetkova, unpublished data). Regular monitoring of frozen sperm has been performed; periodic tests have shown that the quality of sperm does not decrease during storage in the Bank (Tsvetkova et al. 2006; Dokina et al. 2007).

Tsvetkova et al. (2007) reported the protective ability of antifreeze proteins (AFP) or antifreeze glycoproteins (AFGP) extracted from the blood of Barents Sea cod (*Gadus morhua*). Addition of AFGP into cryoprotective media at concentrations from 5 to 25 mg per mL provided a threefold or more increase in fertilizing ability of Siberian sturgeon sperm (Table 2).

Table 2. Dependence of fertilization rate on concentration of antifreeze glycoproteins (AFGP) in cryoprotective media for Siberian sturgeon sperm.

Concentration of AFGP in cryoprotective media, mg/mL	Fertilization rates with post-thawed sperm	
	% relative to fresh sperm	% relative to basic cryoprotective media
5	93±8	355±32
10	78±18	298±69
25	75±21	288±79

Wayman et al. (2008) reported that pallid sturgeon sperm were cryopreserved using varied concentrations of methanol (5, 10, or 15%) and mHBSS at 100 mosM/Kg. Samples were diluted 1:1 (v:v), and were frozen in 0.5-mL straws with an equilibration time of 2 min. The straws were loaded into goblets and placed at the bottom position on aluminum canes and then lowered into a nitrogen-vapor shipping dewar. The average rate of freezing was -22 ^{o}C/min. Straws were thawed at 40 ^{o}C for 9 sec, and used to fertilize ~150 eggs. Highest post-thaw motility (25 - 28%), fertilization rate (91 - 92%), and hatch rate (77%) occurred using 5% or 10% methanol as the cryoprotectant.

Psenicka et al. (2008) analyzed the motility and acrosomal staining characteristics of cryopreserved sterlet sperm in an effort to determine why DMSO when used as a cryoprotectant of sturgeon sperm produced high post-thaw motility, but low fertilization rates as compared to methanol (as shown in previous studies). The sperm were cryopreserved as in Glogowski et al. (2002). After thawing, motility was analyzed using a computer-assisted sperm analysis program, and sperm were stained with SBTI-Alexa Fluor 488. There were no significant differences in the motility characteristics of sperm cryopreserved with methanol or DMSO. However, sperm cryopreserved with DMSO had a significantly higher (12.7%) specific acrosomal staining than did sperm cryopreserved with methanol (6%). The authors postulated that the increased staining indicated damage to the acrosome caused by DMSO during the cryopreservation process. However, overall staining was low, and they could not definitively conclude acrosomal damage was the cause for the low fertilization rates seen with DMSO. The authors stated that the DMSO may be causing preliminary effects that lead to the acrosomal reaction which are not detectable with the current staining technique, but that may be the cause for the reduced fertility. Further studies were warranted.

Horváth et al. (2008) evaluated the relationship between membrane integrity and fertilizing ability for cryopreserved sperm samples that had been previously frozen as reported in Horvath et al. (2005). Sperm from two sturgeon species shortnose sturgeon (*Acipenser brevirostrum*) and pallid sturgeon (*Scaphirhynchus albus*) were tested. Flow cytometry and two

fluorescent dyes were used to assess the membrane integrity of post-thaw sperm. A membrane permeable green fluorescent dye was used to stain all the cells, and then a non-permeable red fluorescent dye was used to counter stain cells with damaged membranes. In most cases membrane integrity correlated with post-thaw motility, but sometimes was independent of fertilization rates.

In conclusion, research of sturgeon sperm cryopreservation has increased dramatically since the publication of the last chapter. Research has progressed from developing basic methods with low fertilization results for only a few species to the refinement of these basic methods for numerous species and resulting in higher levels of fertilization. The newer investigations into alternative approaches to determine sperm quality should also lead to the development of protocols yielding even higher cryopreservation efficiencies.

Cryopreservation Protocols for Paddlefish Sperm

Brown and Mims (1999) were the first to report cryopreservation of paddlefish (*Polyodon spathula*) sperm. Milt was mixed with a cryoprotectant medium containing DMSO (2.4 M) in a ratio of 3:1 (milt:medium; final concentration of DMSO 0.6 M). The medium was composed of 1.6 mL of DMSO, 4.0 mL of trehalose and 4.4 mL of extender. The composition of the extender was 0.205 g $CaCl_2$ H2O, 0.440g $MgCl_2$ $6H_2O$, 0.470 g $NaHCO_3$, 5.115 g KCl, 11.560g NaCl, 20g glucose, 0.200g citric acid, 4.760g HEPES, 2,000 mL double-distilled H_2O, 20 mL KOH, 20mL penicillin-streptomycin, pH 7.6 and 300 mOsmol/Kg. The mixture was stored in 5mL straws, frozen on dry ice for 15 min and then stored in liquid nitrogen. For thawing, straws were immersed in a water bath at 20 ^oC for 15 sec. Motility of post-thawed sperm was 25 to 50%, compared to 100% motility for fresh sperm. Post-thawed samples of two straws (about 5.25 mL of pooled milt and 1.75 mL of medium) were mixed with about 3,500 eggs per trail following standard fertilization procedure (i.e. clay-coating of adhesive eggs) and incubation technique (i.e. McDonald jars). The ratio of motile, post-thawed sperm to egg was about 6.17×10^5 sperm egg^{-1}. Hatching rate was 16 ± 2% from eggs fertilized with post-thawed sperm and was significantly lower than the hatching rate of 90 ± 3% using fresh sperm. The authors postulated that acrosomal damage could have been a major culprit in the low hatching rate and that increasing the post-thaw sperm to egg ratio could mask the low viability of post-thawed sperm. Observation by electron microscopy (Mims and Brown unpublished data) indicated that acrosomal damage probably occurred to paddlefish sperm either during freezing or thawing and probably resulted in premature acrosomal reaction, thus preventing higher rates of fertilization and ultimately higher hatching rates (Figure 2).

Horváth et al. (2006) reported a series of experiments with the goal to improve protocol for sperm cryopreservation of paddlefish. Similar methods that were successfully developed for cryopreservation of sturgeon sperm (Horváth and Urbányi, 2000; Glogowski et al. 2002; Urbányi et al. 2004) were tested on paddlefish sperm. The first experiment was to evaluate the effects of two extenders [mT extender and modified Hank's balance salt solution (mHBSS)] in combination with methanol and DMSO in two concentrations (5 and 10%) on the post-thaw motility and fertilization rates of cryopreserved sperm. The highest post-thaw motility (85 ± 5%) and highest fertilization (80 ± 3%) were observed when sperm was frozen with mT extender and 10% methanol as the cryoprotectant. In sec experiment, 4,000 eggs were fertilized with the pooled samples of five straws per male (total = three males; four replicates per male) of post-thawed sperm representing a volume of 1.25 mL using mT extender and 5% methanol. Hatch

rates were as high as 79 ± 5%. The third experiment was conducted to clarify the role of methanol concentrations. There was no significant difference found among fertilization or hatch rates when either 5 or 10% methanol was used as a cryoprotectant. The authors stated that methanol was found to be a safe and reliable cryoprotectant for freezing of paddlefish sperm and obtaining viable postthaw sperm for consistent fertilization and hatch rates. Further, the experimental protocol is relatively simple and applicable for commercial hatchery production of paddlefish.

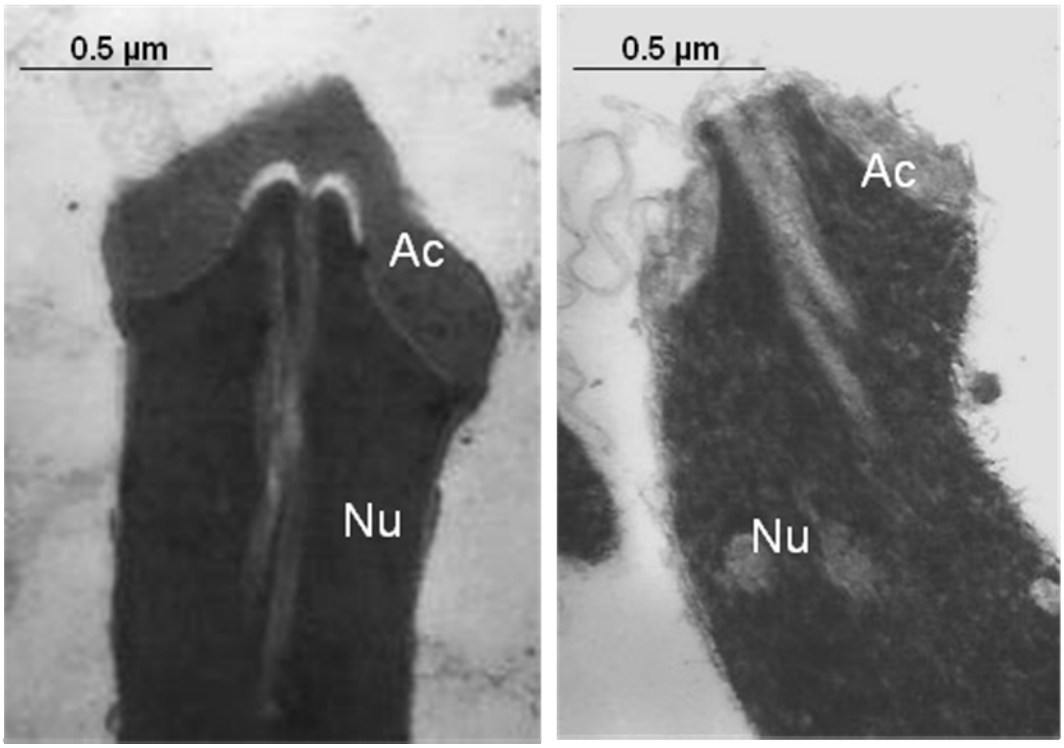

Figure 2. (A) Longitudinal section demonstrating the intact acrosome (Ac) and nucleus (Nu) at the apical tip of fresh paddlefish *Polyodon spathula* spermatozoon, (B) Longitudinal section demonstrating the damaged acrosome (Ac) at the apical end of post-thawed spermatazoon (bar=0.5 µm).

Linhart et al. 2006 compared different percentages (8 and 10%) of cryoprotectants (DMSO and methanol) added to extender 1 (20mM tris pH 8, 30mM sucrose and 0.5mM KCl) or extender 2 (20mM tris pH 8, 50mM sucrose and 0.5mM KCl, dilution 1:1) or non-extended sperm. One mL of the mixture was stored in a 2-mL cryotube. The cryotubes were directly loaded into a pre-programmable PLANER Kryo10 series III freezer at 0 ^{o}C and cooled from 0 ^{o}C to -5 ^{o}C at a rate of -3 ^{o}C/min, from -5 ^{o}C to -15 ^{o}C at a rate of -5 ^{o}C/min, from -15 ^{o}C to -25 ^{o}C at a rate of -10 ^{o}C/min, from -25 ^{o}C to -80 ^{o}C at a rate of -20 ^{o}C/min. The samples were held for 5 min at -80 ^{o}C and then plunged into LN2 for 24 hr. The sperm was thawed in a water bath of 40 ^{o}C for 15 sec. Fertilization rates of 64 to 75% were obtained on post-thawed sperm (3.6 X 10^5 sperm/egg) when sperm was either without dilution or diluted with extender 1 and treated with methanol in concentrations of 8 or 10%. These results were not significantly different compared

with fresh sperm (stored at 3 ^{o}C for 24 hrs). Fertilization rates were only 8-15%, when sperm was frozen with 8 and 10% DMSO.

Bean (2007) reported the hatching rates of paddlefish eggs fertilized with post-thawed sperm previously frozen in different size storage containers. The three cryocontainer sizes were 0.5-mL straws, 2.0-mL cryovials and 4.5-mL cryotubes. Milt was pooled from two males and diluted 1:1 with mT extender and 10% methanol. Containers were placed on a floating tray and were frozen at 3-cm from the surface of LN_2 as described by Horvath et al. (2006) for 3, 4 and 5 min, respectively. Containers were then stored in LN_2 for 24 hrs. Samples in the different containers were thawed by submerging in a 40 ^{o}C water bath for 13, 90 and 120 sec, respectively. Eggs were pooled from two females and weighed into twelve 40 g batches (about 4,000 eggs/batch; 4 replicates/treatment). Five mL of post-thawed sperm from each storage container treatment was added and activated with dechlorinated water. Hatching rates with 0.5-mL straw treatment (control) was 25 ± 6%, with 2.0-mL cryovial treatment was16 ± 12% and with 4.5-mL cryotube was 16% ± 12%. Usage of storage containers of different types and capacities did not have a significant impact on hatching rate of paddlefish.

Horvath et al. (2010) tested the feasibility of using 5-mL straws for the cryopreservation of paddlefish (*Polyodon spathula*) sperm for mass production. In the first experiment the effects of 5% or 10% methanol as a cryoprotectant in combination with cooling times of 5 or 7 min on paddlefish sperm stored in 5-mL straws were evaluated for fertilization and hatching rates. Highest fertilization rate of 48 ± 5 % (mean±SE) and hatching rate of 47 ± 10 % were observed using sperm cryopreserved with 5 % methanol and a 5-min cooling time in liquid nitrogen vapors. However, fertilization and hatching rates were significantly lower with cryopreserved sperm than when fresh sperm (fertilization 77±6 %; hatching 66±13%). In the sec experiment the effects of sperm:egg ratios on fertilization rates were investigated. When fresh sperm was used, fertilization rate was quadratically related to sperm:egg ratio ($y = -13.19x^2 + 55.90x + 38.44$; $r^2=0.82$) and the optimum range of sperm:egg ratios was between 1.379×10^6 and 2.758×10^6. When sperm were cooled for 5 min with 5% methanol, fertilization rate was linear related to sperm:egg ratio ($y=22.51x + 23.26$; $r^2=0.75$) but optimum sperm:egg ratio was not reached. In experiment 3, the hatching rates were not significantly different when using 7.5 mL of post-thawed sperm from three 5 mL straws (69 ± 6%) and 5.0 mL of fresh sperm (77 ± 6%). With cryopreserved sperm, the relationship between the sperm/egg ratios and the hatching rates were best described by a quadratic equation ($y=-29.65x^2 + 119.2x -51.04$, $r^2=0.84$). The authors suggested the volume of cryopreserved sperm should be increased by at least 30% to optimize fertilization and hatching rates.

Horvath et al. 2008 evaluated motility and viability (cell membrane integrity) of cryopreserved sperm from paddlefish. Paddlefish sperm was frozen in mT or mHBSS extenders with methanol or DMSO as cryoprotectants (5 or 10%; v:v). Paddlefish sperm had the highest post-thaw motility (85% ± 2%) and fertilization (80 ± 3%) percentages using 10% methanol; however, the highest viability (59 ± 2%) was observed with mHBSS combined with 5% DMSO. Statistically, post-thaw motility and viability were correlated, but independent of fertilization. The interaction of cryoprotectant and its concentration was significant for fertility, with all fertilization rates higher with 10% methanol than the other combinations and with 10% DMSO yielding significantly the lowest fertilization rate. The decreasing fertility rate with DMSO could be explained by the effect of acrosomal reaction because there is a dramatic increase in osmolaity (i.e. osmotic shock process) and a rapid penetration of the acromosal membrane causing release of acrosin enzyme (Ciereszko et al.1996a,b, 2000; Otomar et al. 2006).

Conclusion

Sturgeon and paddlefish spermatozoa are unique among the male gametes of other fishes because of the presence of a functional acrosome. The majority of the research on cryopreservation of chondrostean sperm had developed protocols that resulted in high motility but low fertilization rates (Horvath et al. 2009). As summarized in this chapter, most of the protocols used dimethyl sulfoxide (DMSO) as the cryoprotectant. However, in the last decade methanol has resulted in significantly higher fertilization and hatching rates than DMSO. Horvath et al. (2009) clearly reported that DMSO greatly increased the osmolality of the extender while methanol caused only a slight increase in osmolality. In addition, when sperm was frozen in hyperosmotic extender (i.e. original Tsvetkova extender as described in Tsvetkova et al. 1996) containing either DMSO or methanol as cryoprotectant, poor fertilization also occurred. Therefore, iso-osmotic freezing diluents (i.e. combined extender and cryoprotectant) are necessary to use for cryopreserving chondrostean sperm in order to obtain consistent fertilization success.

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Cryopreservation of Sperm of the Endangered Razorback Sucker

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Introduction

The native fishes of the Colorado River system of the southwestern United States have declined substantially during the past century and their prospects for survival are not good. Numerous factors including introduction of exotic species and construction of dams have extensively disrupted the natural habitat of these fishes and have resulted in major changes in the aquatic fauna. The razorback sucker *Xyrauchen texanus* was once abundant and widely distributed throughout the Colorado River and its tributaries, but is now found commonly only in the middle Green River and Lake Mohave, below Hoover Dam (Minckley et al. 1991, Modde et al. 1996). Razorback suckers are threatened with extinction because of limited recruitment into the adult size classes, and in 1991, received a formal listing under the Endangered Species Act (U.S. Fish and Wildlife Service 1991). Because of the lack of natural recruitment, artificial propagation and reintroduction are emphasized for the conservation, management and recovery of this species. Methods that enable the transfer of gametes from wild populations to hatchery broodstock (e.g. Cloud et al. 1990) are needed to aid in this recovery program.

Recommendations identified by a multi-agency management team include the stocking of Lake Mohave with razorback suckers to replace the aging population (Figure 1). Razorback suckers are currently maintained for research and stocking at the U.S. Fish and Wildlife Service (USFWS) Dexter National Fish Hatchery and Technology Center (Johnson and Jensen 1991) and at the Willow Beach National Fish Hatchery (WBNFH). Management of razorback suckers has included production and distribution of fertilized eggs and larval fish in protected backwaters adjacent to Lake Mohave (Mueller 1995).

Photograph by T. Tiersch

Figure 1. Male razorback sucker collected from Lake Mohave in 1995. Note the distinctive dorsal keel. Many razorback suckers in this population carry lesions or growths (such as on the tail of this fish) that may be related to advanced age.

Storage and cryopreservation of sperm is an effective management tool for conserving genetic resources of threatened and endangered populations. This technology offers advantages by providing genes from wild populations for hatchery broodstock, greater control in breeding

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programs, and the ability to store large amounts of valuable germplasm for long periods. Gamete storage is an effective way of solving hatchery-related problems of differential maturation of broodstock by allowing flexibility in spawning time. Our purpose in this work was to improve and integrate gamete collection, storage and cryopreservation for enhancement of recovery efforts for razorback suckers (Carmichael et al. 1996, Figiel et al. 1996, Tiersch et al. 1997, 1998). The objectives were to: 1) develop methods for collection of sperm allowing integration with established sampling programs; 2) characterize sperm motility and duration; 3) develop methods for refrigerated storage of sperm; 4) develop methods for the cryopreservation of sperm; 5) fertilize eggs with cryopreserved sperm, and 6) investigate methods for incubation of eggs.

Collection of Gametes

We collected fish from a 17-km section of the Colorado River between WBNFH and Hoover Dam on upper Lake Mohave during the 1994 to 1996 spawning seasons. We integrated our procedures with sampling protocols established by the USFWS: fish were weighed, measured, tagged with passive integrated transponders and general condition was recorded. Sperm were collected immediately after USFWS protocols were completed or fish were kept in live-wells and hatchery tanks. To collect sperm, males were held head down with ventral surface up, and were wiped to remove excess water and debris. The head was raised, and the anal fin was held against the caudal peduncle, exposing the vent. To initiate semen flow, we rotated the ventral surface of the fish downward, and applied gentle pressure behind the pectoral fins, which minimized handling of fish and the contamination of semen with feces and urine often associated with application of pressure along the belly toward the vent. Unless kept undiluted for use in other studies, sperm were diluted with calcium-free Hanks' balanced salt solution (C-F HBSS) and stored on ice for transport to WBNFH. In the laboratory, we stored refrigerated (4 °C) sperm samples in loosely capped tubes. Sperm collection was completed within 1 min and did not interrupt the established sampling protocols.

Characterization of Sperm Motility and Duration

For estimation of motility, 2 µL of sperm were placed on a microscope slide and diluted with 20 µL of water collected from Lake Mohave (21 mOsmol/Kg). Sperm became motile and initiated rapid swimming when diluted. The percentage of sperm swimming actively in a forward direction was estimated using dark-field microscopy at 200-× magnification. The duration of motility was divided into three periods: 1) time required to reach maximum motility after addition of water; 2) duration of maximum motility, and 3) time until complete cessation of motility.

The time required to reach maximum motility was 3 sec after the addition of water (the minimum time at which accurate estimates of motility could be made was 2 sec). Maximum motility was maintained for 16 ± 8 sec (mean ± SD), and the time until complete cessation of motility was 70 ± 32 sec after the addition of water.

Refrigerated Storage of Sperm

We performed two experiments on the motility retention of razorback sucker sperm during storage at 4 °C. In the first, we compared motility of undiluted sperm and sperm diluted

with an equal volume of C-F HBSS. In the sec, we compared motility of sperm stored in three dilutions: 1 part semen to 1, 3, or 7 parts C-F HBSS. Sperm were collected as described above, diluted 1:1 in the field, delivered within 1.5 hr, and aliquots were diluted beyond 1:1 at WBNFH. We chose six high quality samples (>95% motility) and placed these in loosely capped 15-mL tubes and stored them upright at 4 °C. We estimated motility immediately after final dilution, and daily for 3 d until samples were shipped by commercial airline to Louisiana State University, where daily estimates were continued until all samples became non-motile.

In the first experiment, there was a significant difference in sperm motility within 24 hr between sperm diluted (1:1) with C-F HBSS and undiluted sperm (t-test; $t = -6.45$, $P < 0.0001$). All undiluted sperm samples became non-motile within 72 hr. About half (45%) of the diluted sperm samples retained at least 60% motility for 5 d. In the sec experiment, dilution of sperm with different proportions of C-F HBSS did not affect sperm motility on d 3 (ANOVA, $P = 0.1815$), or d 8 of the experiment (ANOVA, $P = 0.5958$). Overall, sperm motility was highest on d 1 (73 ± 10%) and decreased through d 8 (2 ± 6%). Sperm samples retained an average motility of greater than 15% motility for 6 d, although nine of the fifteen samples appeared degraded and were non-motile after 3 d.

Cryopreservation of Sperm

We examined the effects of six cryoprotectants on sperm motility: dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), glycerol, methanol (MeOH), propylene glycol and ethylene glycol. Cryoprotectants minimize damage to sperm cells during the freezing and thawing processes (Jamieson 1991), but cryoprotectants can have toxic effects on sperm as well. In the first experiment, we examined the effects of 5 and 10% of DMSO, MeOH, DMA and glycerol on post-thaw sperm motility of four males. Cryoprotectants were dissolved to the appropriate concentration and added to sperm samples. Samples were allowed to equilibrate for 90 min at room temperature (~25 °C) before the straws (0.5 mL) were placed into goblets and into shipping dewars (Taylor-Wharton models CP-35 and CP-65) for freezing. In the sec experiment, we examined the effects of 5 and 10% of DMSO, ethylene glycol, and propylene glycol, and 10 and 20% of MeOH on post-thaw sperm motility of five males. We followed the same procedures as above except that sperm samples were allowed to equilibrate for 8 min before the straws (0.5 mL) were placed into the dewars. In each experiment, sperm samples had 80 to 95% initial motility and were held frozen for at least 24 hr.

In the first experiment, cryoprotectant (and concentration) influenced post-thaw motility. Motility of sperm cryopreserved with 10% MeOH was significantly higher (ANOVA, $P < 0.01$) than the motility of sperm cryopreserved with 5% MeOH, or either concentration of DMSO, DMA, or glycerol. Similarly, in the sec experiment, motility of sperm cryopreserved with 10% MeOH (24 ± 2%) was significantly higher (ANOVA, $P < 0.01$) than the motility of sperm cryopreserved with 20% MeOH, or either concentration of DMSO, propylene glycol, or ethylene glycol.

Fertilization of Eggs Using Cryopreserved Sperm

We cryopreserved sperm from three male razorback suckers with 10% MeOH (pre-freeze motility was 50% for each male). Sperm samples were aspirated into straws (0.5 mL) and placed into shipping dewars for freezing. Two sperm samples from each male were thawed after 24 hr

and motility was estimated. These sperm samples were mixed immediately with 500 to 600 eggs from each of two females so that there were a total of six fertilization attempts (a 0.5-mL sperm sample from each male for eggs from each female). Additionally, we fertilized eggs from both females using refrigerated sperm from two males (90 and 95% motility) to serve as controls. After 96 hr, we determined the percentage of developing eggs. Refrigerated sperm yielded fertilizations of 50% (female one) and 33% (female two). Cryopreserved sperm yielded fertilizations of 35% (female one) and 18% (female two). The mean percent motility of thawed sperm samples was 18% (n = 6).

Incubation of Eggs

We used polyethylene storage bags (Ziplock® brand) for the fertilization of eggs and the incubation and hatching of embryos. We placed 200 to 2,000 eggs within each bag (although best results were obtained with less than 500 eggs). Eggs were fertilized by addition of either refrigerated or cryopreserved sperm and 50 mL of Colorado River water. After 30 sec, water volume was increased to 250 mL for water hardening of the eggs. Bottled oxygen was used to supplement the air within the bags. The water (23 °C) was exchanged twice daily, and fungal growth was controlled by manual removal of hyphae-infected eggs. Use of these bags enabled us to keep experimental groups of eggs separated and to replicate large number of trials. This was especially important when examining multiple crosses in large experiments. Additionally, these bags provided ease in transportation of embryos and fry, and the transparency of the bags permitted observations on embryo development and treatment effects.

Discussion and Conclusions

We were able to combine research on the collection, storage, and cryopreservation of sperm with an established sampling program for razorback suckers. Our studies demonstrated that collection of sperm was rapid and did not disrupt fish sampling procedures. Collection of sperm was performed in the boat during routine data collection and tagging, and was quickly mastered by sampling crews. Sperm of razorback suckers became active when diluted in river water and swam vigorously for 20 sec, losing all motility at about 70 sec after dilution. Given this relatively short time of maximal activity, care should be taken to ensure good, early mixing of gametes during artificial spawning of razorback suckers.

Refrigerated storage of sperm is an effective method for management of razorback sucker broodstock in that it allows flexibility in spawning of females. Calcium-free Hanks' balanced salt solution allowed refrigerated storage of razorback sucker sperm for at least 7 d. Bacterial contamination may have caused degradation of sperm samples after that time. Potentially, sperm survival could be prolonged by addition of antibiotics to inhibit bacterial growth (Stoss et al. 1978, Stoss and Refstie 1983). Methanol appeared to be the most effective cryoprotectant for razorback sucker sperm. Sperm cryopreserved with 10% methanol had higher post-thaw motility than did sperm with other cryoprotectants. Although sperm motility was reduced because of the cryopreservation process, the sperm were useful for fertilization of eggs. Razorback suckers produced in 1996 at WBNFH with cryopreserved sperm have been reared and currently (as the first edition of this volume went to press in 2000) are maintained in ponds at the Dexter National Fish Hatchery and Technology Center.

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Annotated Bibliography of the Developments in the Last Decade

In the Colorado River system, four of 14 native fish species are listed as endangered, including the Colorado pikeminnow *Ptychocheilus lucius*, razorback sucker *Xyrauchen texanus*, bonytail chub *Gila elegans*, and humpback chub *Gila cypha*. In 1988 a conservation program named as "The Upper Colorado River Endangered Fish Recovery Program" was established under a cooperative agreement among the US Fish and Wildlife Service, the Arizona Game and Fish Department, the Colorado Division Of Wildlife, and the Utah Department Of Wildlife, and was extended through 2013 (*www.fws.gov/mountain-prairie/crrip/*). The effort of this program has focused on removal of non-native game fish species, habitat restoration, breeding, and genetics. The 2006 annual report of this program indicated that native species are still rare in many of the study areas, and listed possible causes including habitat loss through dam construction, irrigation diversions in the Colorado basin, and competition with non-native fishes. A gamete cryopreservation program is urgently needed for conserving these endangered species. A search for references found a new publication on sperm cryopreservation of Colorado pikeminnow (Tiersch et al. 2004). More investigation is urgently needed for the conservation and recovery of these endangered species.

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New Chapter

Current Status of Sperm Cryopreservation in Siluriform Catfishes

Ana T. M. Viveiros

Introduction

Catfishes belong to the class Actinopterygii (ray-finned fishes) and the Order Siluriformes which contains 33 families. During the past decade, 6 families and 9 species of this Order were subjects of cryopreservation studies (Table 1). The three most important families used worldwide for aquaculture are Clariidae, Ictaluridae, and Pangasiidae. The clariids are cultured predominantly in South Africa (Steyn et al. 1985), the ictalurids (mainly channel catfish *I. punctatus*) are cultured in the United States (Christensen and Tiersch 2005) and the pangasiids are cultured throughout Southeast Asia (Withler 1982). Because of overfishing, habitat degradation, and pollution, some catfish species, such as *Pangasius gigas* (Mongkonpunya et al. 2000a) and *Pangasius larnaudii* (Kwanton and Bart 2006) are in decline in the wild.

Table 1. List of fresh and brackish water siluriform species that have been studied for sperm cryopreservation studies performed since 1998, and the method of semen collection.

Family	Species	Common name	Collection method
Bagridae	*Mystus nemurus* (=*Hemibagrus nemurus*)	Asian redtail catfish	Crushed testis
	Pelteobagrus fulvidraco	yellow catfish	Crushed testis
Clariidae (airbreathing catfishes)	*Clarias gariepinus* (=*Clarias lazera*)	North African catfish	Crushed testis
Ictaluridae (North American freshwater catfishes)	*Ictalurus furcatus*	blue catfish	Crushed testis
	Ictalurus punctatus	channel catfish	Crushed testis
Pangasiidae (shark catfishes)	*Pangasius gigas* (=*Pangasianodon gigas*)	Mekong giant catfish	Hand-stripping
	Pangasius hypophthalmus (=*P. sutchi*)	striped catfish, sutchi catfish	Hand-stripping
	Pangasius larnaudii	black ear catfish, spot pangasius	Hand-stripping
Pimelodidae (long-whiskered catfishes)	*Pseudoplatystoma corruscans* (=*P. coruscans*)	spotted sorubim	Hand-stripping
Siluridae (sheatfishes)	*Silurus glanis*	wels catfish, European catfish	Hand-stripping

Viveiros, A. T. M. 2011. Current Status of Sperm Cryopreservation in Siluriform Catfishes. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 387-397.

The majority of the catfish species reared in captivity exhibit some form of reproductive dysfunction, leading to a reduced semen production or low quality in most males. Reproductive hormones have successfully been used to induce spermiation and increase semen volume in several species. However, in some catfish species (mostly clariids and ictalurids), the response of males to hormone-induced spermiation remains poor, and semen is not released by hand stripping (Table 1). In practice, males of these families are sacrificed, testes are macerated and intratesticular sperm is spread over the eggs. For a review of semen collection in catfish species, please refer to Viveiros (2003).

Gamete cryopreservation and artificial fertilization offer benefits for genetic improvement of commercially produced fishes as well as for conservation purposes. Conservation of genetic variability of wild catfish populations is also of importance, as domestication can lead in short time periods to the formation of phenotypically altered strains with reduced genetic variability. The use of limited numbers of broodstock without adding genes from unrelated fish to successive generations is responsible for the occurrence of genetic drift and the founder effect in most domesticated *Clarias gariepinus* populations (Van der Walt et al. 1993). In attempts to maximize the use of sperm from single males, to develop stocks with improved traits for selection programs, and to preserve species from extinction, sperm cryopreservation protocols have been developed for several catfish species. The direct comparison of information in many published protocols is, however, difficult. The reported post-thaw viability, even for a single species, is highly heterogeneous, some reports are incomplete, and given that only positive results are usually published, the true variability of results remains unknown. Because of these difficulties, development of reliable cryopreservation protocols for fish sperm is often practiced on a species-by-species basis.

To assist in sharing knowledge, emphasis should be placed on standardization on each step in a procedure: semen collection, dilution ratio, extenders, cryoprotectant agents (CPA), equilibration time, cooling rates, thawing rates, and sperm-to-egg ratios in fertilization tests (Mongkonpunya et al. 2000a). Before any protocol is adopted, methods for evaluation should be carried out for each step. Motility tests are mostly used to assess sperm viability after thawing, however, in experiments with *C. gariepinus* (Viveiros et al. 2000), *P. gigas* (Mongkonpunya et al. 2000a) and *Ictalurus furcatus* (Lang et al. 2003), post-thaw samples with little to no motile cells still resulted in successful fertilization. Furthermore, in *P. larnaudii*, while post-thaw sperm viability (eosin/nigrosin staining) was 50%, the fertilization rate was 70% (Kwanton and Bart 2006). Therefore, fertility or hatching rates should be the method used to evaluate post-thaw sperm quality whenever possible. On the other hand, when fertilization is included in the post-thaw evaluation, sperm-to-egg ratio must be optimized. Using an excess of sperm cells for fertilization can mask the quality of fresh or cryopreserved semen, making comparison of protocols difficult. This chapter reviews the knowledge concerning semen cryopreservation of catfish species, with emphasis on research published over the last decade and available through PubMed, ISI Web of Knowledge, or Google Scholar.

Freezing Protocols

A complete-freezing protocol combines cooling rate, the number of steps involved during cooling (program type) and temperature at which plunging into liquid nitrogen (LN_2) occurs. The combination of these three elements determines whether or not viable cells can be frozen to temperatures that permit indefinite storage. Some background information on cryobiology is

necessary to understand the possible causes of injuries that can occur during the freezing and thawing processes and how to prevent them. For details on cryobiology, readers should refer to Mazur (1970, 1977), Leibo (2000), and Viveiros (2005) among others. It is well known that injuries can be caused by slower- or faster-than-optimal cooling and thawing rates, and that the level of injury can be affected by the choice of cryoprotectant and the composition of extenders.

To reduce intracellular ice formation during fast freezing, two- and three-step programs have been used in catfish semen freezing protocols. To freeze using a two-step program, programmable freezers, or nitrogen (N_2) vapor in a styrofoam frame or a shipping dewar (dry-shipper) can be used. In both situations, semen is frozen at an initial slow cooling period (mostly to -80 °C) followed by rapid cooling as samples are plunged into LN_2 for final storage. Please refer to Table 2 (next page) for rates that are commonly used.

In *C. gariepinus*, a programmable freezer was used to freeze semen at -2, -5 and -10 ºC/min in combination with different temperatures at plunging into LN_2 (-25 to -70 ºC). Hatching rates equal to frozen semen were obtained by samples frozen at -5 ºC/min to -45 to -50 ºC and at -10 ºC/min to -55 ºC (Viveiros et al. 2000). The same researchers repeated this experiment measuring freezer and semen temperature during the freezing program. It was observed that cells cooled at a rate of -2 to -5 ºC/min survived plunging at a semen temperature of -38ºC, while cells cooled at -10 ºC/min did not. At -10 ºC/min, high post-thaw sperm survival (~76% control hatching) was obtained only when plunging occurred at a semen temperature of -51 ºC. It appears that, at a cooling rate of -10 ºC/min, and certainly at faster rates, cell dehydration lags behind and osmotic equilibration is not reached or approached (Viveiros et al. 2001). Besides *C. gariepinus*, programmable freezers have been used to cryopreserve semen of *I. punctatus, P. larnaudii*, *P. gigas, P. hypophthalmus* and *Silurus glanis* over the past decade (Table 2).

Styrofoam frames floating on the surface of LN_2 to produce a two-step freezing program have been used with success in *C. gariepinus* (Miskolczi et al. 2005), *I. furcatus* (Bart et al. 1998) and *S. glanis* (Ogier de Baulny et al. 1999). When *C. gariepinus* semen was frozen in N_2 vapor, although no difference on fertilization or hatching rates was observed among straw sizes and fresh control samples, haploid embryos were found in malformed larvae hatched from eggs fertilized with cryopreserved semen. The authors hypothesized that the genome of these fertilizing spermatozoa was damaged during freezing, and thus these cells could still move and fertilize eggs but could not be incorporated into the diploid genome of the developing embryo (Miskolczi et al. 2005). A variation of this freezing method can be obtained with a N_2-vapour shipping dewar that can be used while on boats and freeze sperm from wild fish. Shipping dewars have been used with success in *P. gigas* (Mongkonpunya et al. 2000a) and *Pseudoplatystoma corruscans* (Carolsfeld et al. 2003). A description of freezing procedures in the field using shipping dewar can be found in Wayman and Tiersch (2000).

The three-step freezing program consists of an isothermal holding period added to the end of the slow cooling period before plunging. There are some studies on *C. gariepinus* that describe when a three-step program improves post-thaw survival and when it does not, compared to a two-step program. Semen was frozen at -5ºC/min to -35 and -40ºC in a programmable freezer and plunged into LN_2. The hatching rates produced by these samples were 0%, but when samples were held for 5 min at -35 or -40ºC before plunging, the hatching rates increased to values similar to that of fresh semen (Viveiros et al. 2000). In a subsequent study, the same authors measured freezer and semen temperature during freezing. It was observed that when the freezer was at -35 ºC, semen was at -13 ºC, but decreased to -33 ºC after 5-min of holding.

Table 2. Freezing protocols that yielded the highest post-thaw semen quality of catfish species in studies performed since 1998.

Type of Freezer	Freezing Program	Container	Species	Reference
Programmable	-4°C/min to -9°C; -11°C/min to -80°C	1.8-mL cryotube	*S. glanis*	Linhart et al. 2005
Programmable	-4°C/min to -4°C; -11°C/min to -80°C	0.25-mL straw	*C. gariepinus*	Urbanyi et al. 1999, Horvath and Urbanyi 2000, Urbanyi et al. 2000
Programmable	-5°C/min; held at -40°C for 5 min	1.0-mL cryovial	*C. gariepinus*	Viveiros et al. 2000, Viveiros et al. 2001, Viveiros and Komen 2008
Programmable	-8°C/min from -5° to -80°C	1.8-mL cryotube	*C. gariepinus*	Rurangwa et al. 2001
Programmable	-10°C/min	2-mL cryotube	*P. gigas*	Mongkonpunya et al. 2000a
Programmable	-10°C/min to -80°C	0.25-mL straw	*P. larnaudii*	Kwantong and Bart 2006
Programmable	-10°C/min to -80°C	0.25-mL straw	*P. hypophthalmus*	Kwantong and Bart 2003 Kwantong and Bart 2009
Programmable	-45°C/min; held at -80°C for 10 min	0.5-mL straw	*I. punctatus*	Christensen and Tiersch 2005
N_2 vapor in frames	3 min at 3-4 cm above LN_2 surface	0.25-, 0.5-, 1.2-mL straws	*C. gariepinus*	Miskolczi et al. 2005
N_2 vapor in frames	3 min at 3 cm above LN_2 surface	0.5-mL straw	*C. gariepinus*	Kovács et al. 2010
LN_2 vapor in frames	15 min at 6.5 cm above LN_2 surface	0.5-, 1-mL straws	*I. furcatus*	Bart et al. 1998
LN_2 vapor in frames	10 min at 6 cm above LN_2 surface; 5 min on LN_2 surface	2-mL cryotube	*P. fulvidraco*	Pan et al. 2008
N_2 vapor	20 min 3 cm above LN_2 surface	0.5 mL straw	*S. glanis*	Ogier de Baulny et al. 1999
N_2 vapor (dewar neck)	Until semen reached -80°C	2-, 5-mL cryotubes	*P. gigas*	Mongkonpunya et al. 2000b
N_2 vapor (commercial chamber)	-16°C/min to -140°C	0.5-mL straw	*I. furcatus*	Lang et al. 2003
N_2 vapor (shipping dewar)	Samples remained in shipping dewar for $\leq$ 1 wk	0.5-mL straw	*P. corruscans*	Carolsfeld et al. 2003
Crushed ice + dry ice	Crushed ice 5 min, dry ice 5 min	5-mL vial	*M. nemurus*	Muchilin et al. 2004

Similarly, during a 5 min holding period at -40 ºC, semen temperature fell from -21 ºC to -38 ºC. The decrease in semen temperature during the holding period before plunging was followed by an increase in the hatching rate of these samples after thawing. During the holding period, while the freezer temperature remained constant, the semen temperature continued decreasing until equilibrium with the freezer was achieved or until plunging occurred. Thereby, extra time was provided for water efflux and to eliminate spatial temperature gradients within the semen sample. When a faster cooling rate of -10 ºC/min was used in combination with a three-step program, semen could be plunged into LN_2 at a semen temperature of -36 ºC instead of -51 ºC (two-step program) to produce high hatching rates (Viveiros et al. 2001). For the sake of clarity, the use of a holding period is not always beneficial. At the slowest cooling rate tested, -2 ºC/min, the addition of holding periods reduced sperm viability. It seems that at such a slow rate, the cells were already in osmotic equilibrium with the surrounding medium. Remaining at a constant low temperature would expose the cells to a highly concentrated solute and may force excessive dehydration and shrinkage (Viveiros 2005). A simple freezing method to produce a three-step program is described for *Mystus nemurus* sperm (Muchilin et al. 2004). Vials containing 5 mL of diluted sperm were placed on crushed ice for 5 min, then on dry ice for another 5 min and finally plunged into LN_2. A freezer was not used and yet post-thaw motility of 50% was achieved.

The high temperature (-36 to -38 ºC) at which plunging into LN_2 occurred, as observed in Viveiros et al. (2001), merits special attention. While most of the research on semen cryopreservation focuses on freezing injuries caused by fast cooling (i.e., intracellular ice formation), other sources of injuries caused by slow cooling have received less attention. In fact, the formation and dissolution of intracellular ice are lethal and no hatching is obtained when semen is frozen too fast or is plunged into LN_2 before dehydration has reached the level necessary to preclude intracellular freezing. This is why most protocols use a low plunging temperature for semen such as -80 °C and even -140 °C for different catfish species (Table 2). However, in *C. gariepinus*, decreasing hatching rates were obtained when semen temperature at plunging was below -50 ºC. The authors speculated that below -50ºC and at relatively slow cooling rates, sperm cells were damaged by too long exposure to high concentrations of electrolytes or continued osmotic shrinkage. According to these results, catfish semen should be cooled at a rate of -2, -5, or -10 ºC/min to a freezer temperature of -40 ºC and plunged into LN_2 as soon as the semen temperature reaches -38 ºC (Viveiros et al. 2001). One should keep in mind, however, that the suggested protocol is valid only when 1-mL cryovials are used. Cryovials are shorter and wider, and consequently have a smaller volume-to-surface area ratio, compared to straws. While cooling occurs more uniformly among sperm cells inside a straw, different temperatures can be measured inside a cryovial. Lower temperatures are found among the cells closer to the cryovial wall, while higher temperatures are found more at the center (Viveiros 2005). Similar research on optimal plunging temperature should be carried out with semen of other catfish species and using other semen containers such as straws of volumes ranging from 0.25- to 5.0-mL.

The warming rate during thawing of the semen is also of importance. The effects of warming rate on cell survival will depend on the cooling history. Cells cooled too rapidly will have large intracellular ice crystals. Cells cooled more slowly, but still relatively fast could have small intracellular ice crystals. During slow warming these crystals could grow in a process called recrystallization, which could damage the cells. These cells could therefore be preserved by rapid thawing. When cells are cooled very slowly intracellular ice formation could be insignificant, and a high warming rate may not be needed. Actually, these cells are probably very

much dehydrated and shrunk and could be damaged by rapid thawing because of a too rapid rehydration (Leibo 1976). In general, frozen catfish semen has been mostly thawed in a water bath at 25-50 ºC from 5 sec to 5 min.

Cryoprotectant Agents

Although the survival of the cells during freezing and thawing can be maximized by optimizing protocols, post-thaw recovery will be unacceptably low if cryoprotectant agents (CPA) are not added to the freezing medium. The discovery that glycerol could prevent the damaging effects of slow cooling and thawing on spermatozoa and red blood cells (Polge et al. 1949), renewed interest in banking of living cells and tissues at low temperatures. Since then, many other permeating compounds have been found to protect living cells against some of the damage caused by freezing. The most common CPA used over the past 10 years to protect catfish sperm are dimethyl sulfoxide (DMSO), methanol, and dimethyl acetamide (DMA) mostly at 10% (v: v), varying from 5 to 15% (Table 3, next page).

Methanol has the smallest molecular weight and thus the fastest cellular permeation when compared to other permeating compounds such as DMSO, DMA, and glycerol. However, whether these compounds need to permeate cells to be effective is still unclear. Experiments using different ways of blocking CPA permeation (such as the addition of copper ions in the medium, a short equilibration time of 30 sec, or equilibration at temperatures that a cell is not permeable to the CPA) have demonstrated post-thaw motility above 40%. Perhaps, the ability of a cell to survive freezing depends more on protection of the cell surface than on protection of the cell interior (Mazur 1970). For most of the circumstances, equilibration time can be set at 15 to 30 min, but it varies depending on the type and concentration of CPA being used (Wayman and Tiersch 2000).

In a preliminary experiment carried out at Wageningen University, fresh *C. gariepinus* semen was incubated (4 ºC) with DMSO, glycerol, or methanol at concentrations of 0-25% for 0-60 min prior to fertilization. It was found that DMSO could be used at a maximum of 15% with a 5-min equilibration and glycerol at 5% with a 5-min equilibration, without reducing fertilizing ability. Longer equilibration periods or higher DMSO or glycerol concentrations affected sperm viability and significantly decreased the hatching rates (Viveiros 2005). Similarly, in *C. gariepinus* 5% glycerol in combination with a 2-min equilibration significantly reduced the post-thaw sperm viability (Horváth and Urbanyi 2000). In other preliminary experiments, fresh semen incubated with methanol at concentrations as high as 20% for 60 min, did not affect sperm fertilizing capacity. Only when methanol was used at 25%, did the sperm fertilizing capacity decrease. When experiments on semen cryopreservation followed, it was confirmed that methanol was a more suitable CPA for freezing of *C. gariepinus* semen compared to DMSO (Viveiros et al. 2000).

However, other researchers have observed for the same species, that the highest post-thaw motilities were yielded by semen frozen in DMSO compared to those samples frozen in DMA, glycerol, methanol, ethylene glycol, or propylene glycol (Horvath and Urbanyi 2000) and similar post-thaw motility and fertility were observed between semen frozen in DMSO or DMA (Urbanyi et al. 2000).

Table 3. Freezing media that yielded the highest post-thaw semen quality for catfish species in studies performed since 1998.

Cryoprotectant	Extender	Post-thaw semen quality	Species	Reference
DMSO	0.9% NaCl	67% motility; 70% fertilization of control; 38% live cells eosin:nigrosin	*P. larnaudii*	Kwantong and Bart 2006
DMSO	0.9% NaCl	81% fertility of control	*P. hypophthalmus*	Kwantong and Bart 2003
DMSO	0.9% NaCl	73% fertility of control	*P. hypophthalmus*	Kwantong and Bart 2009
DMSO	1.2% NaCl + Tris-HCl; pH 7.0	50-61% motility; ~95% hatching of control; 7-17% intact membrane	*S. glanis*	Linhart et al. 2005
DMSO	0.9% NaCl or calcium-free HBSS; ~280-300 mOsmol/Kg	~100% fertility of control	*P. gigas*	Mongkonpunya et al. 2000a
DMSO	0.9% NaCl or HBSS; ~300 mOsmol/Kg	--	*P. gigas*	Mongkonpunya et al. 2000b
DMSO	Powdered milk + HBSS; pH 7.4	32% fertility of control	*I. furcatus*	Bart et al. 1998
DMSO	Mounib + 10% egg yolk	70% motility; ~60% hatching of control	*C. gariepinus*	Rurangwa et al. 2001
DMSO	6% fructose; pH 7.7	25% motility; 100% fertility of control	*C. gariepinus*	Urbanyi et al. 1999;
DMSO	6% fructose; pH 7.7	44% motility; ~80% hatching of control	*C. gariepinus*	Horvath and Urbanyi 2000
DMSO or DMA	6% fructose; pH 7.7	50% motility; 90-95% fertility of control	*C. gariepinus*	Urbanyi et al. 2000
DMA	Mounib + 10% egg yolk; pH 7.8	55-60% motility; 45-50% intact membrane and mitochondrial activity	*S. glanis*	Ogier de Baulny et al. 1999
DMSO or methanol	6% fructose; pH 7.7	74-99% fertility of control; 73-90% hatching of control; 33-44% malformed larvae	*C. gariepinus*	Miskolczi et al. 2005
Methanol	6% fructose; pH 7.7	~100% fertility of control	*C. gariepinus*	Kovács et al. 2010
Methanol	5% glucose + 15% powdered milk	60-80% motility	*P. corruscans*	Carolsfeld et al. 2003
Methanol	HBSS; 295 mOsmol/Kg	48% motility	*I. punctatus*	Christensen and Tiersch 2005
Methanol	HBSS; 290-300 mOsmol/Kg	11-50% motility; 54-83% neurulation	*I. furcatus*	Lang et al. 2003
Methanol	Ginzburg fish Ringer; pH 7.6; 244 mOsmol/Kg	~100% hatching of control	*C. gariepinus*	Viveiros et al. 2000, 2001, Viveiros and Komen 2008
Methanol	Ringer; pH 7.5; 260 mOsmol/Kg	92% hatching of control	*P. fulvidraco*	Pan et al. 2008
Methanol	Ringer; pH 7.9	58% motility	*M. nemurus*	Muchilin et al. 2004

-HBSS, Hanks' balanced salt solution (g/L): $CaCl_2$ 0.14; NaCl 8.0; KCl 0.4; $MgSO_4.7H_2O$ 0.20; $Na_2HPO_4.7\ H_2O$ 0.06; KH_2PO_4 0.03; $NaHCO_3$ 0.17; glucose 1.0. -Mounib solution (g/L): reduced glutathione 2.0; $KHCO_3$ 10.0; sucrose 42.0. Ginzburg fish Ringer (g/L): NaCl 6.5; KCl 0.25; $CaCl_2$ 0.3; $NaHCO_3$ 0.2.
-Ringer (g/L): NaCl 7.5; $NaHCO_3$ 0.2; KCl 0.2; $CaCl_2.2H_2O$ 0.2; glucose 5.
-Intact membrane: not stained with propidium iodide; Mitochondrial activity: stained with Rhodamine 123.

In other catfish species, the same contrast in results have been reported. Semen of *I. furcatus*, frozen in DMSO produced higher post-thaw fertilization rate compared to samples frozen in methanol (Bart et al. 1998). However, fresh semen of the same species exposed to methanol for 30 min yielded the highest sperm motility rates compared to samples exposed to glycerol, DMSO, or DMA. Based on these results, methanol was chosen for use in subsequent cryopreservation trials (Lang et al. 2003). In *S. glanis*, DMA was the best CPA compared to DMSO, glycerol, methanol, or propylene glycol (Ogier de Baulny et al. 1999). Semen samples of *P. hypophthalmus* frozen in DMSO produced the highest post-thaw fertilization, compared to samples frozen in DMA, ethylene glycol, or methanol (Kwantong and Bart 2003). In *M. nemurus*, samples frozen in methanol yielded the highest post-thaw motility compared to samples frozen in ethanol, glycerol, or DMSO (Muchlisin et al. 2004).

Non-permeating compounds such as sugars (e.g., glucose and fructose) and proteins (e.g., powdered milk and egg yolk) are frequently added to the freezing medium and are known for their membrane-stabilizing activities (Wayman and Tiersch 2000). Egg yolk has been used in combination with Mounib solution for *C. gariepinus* (Rurangwa et al. 2001) and *S. glanis* semen (Ogier de Baulny et al. 1999), while powdered milk has been used in combination with glucose for *P. corruscans* semen (Carolsfeld et al. 2003).

The differences in catfish sperm sensitivity to different CPA observed in the literature (Table 3) may be due to different protocols used to incubate semen (such as extender composition, dilution ratio, temperature during equilibration, or semen container) or differences in methods to assess sperm motility or fertility (such as sperm-to-egg ratio). Furthermore, it is possible that differences in seminal plasma composition exist between wild and domesticated catfish, or among domesticated catfish reared under different conditions, and that these differences may have influences on sperm sensitivity to CPA.

Final Remarks

The efficacy of cryopreservation is dependent on sperm quality before freezing. Semen should be collected under hygienic conditions to avoid contamination by urine, mucus, or water. In some freshwater teleost fishes, sperm is held within the testis in an immotile state by the ionic concentration in the seminal plasma. Sperm immotility is said to be attributed to the presence or reductions of ions such as K^+, Na^+, Ca^{++} and Mg^{++}. Sperm motility in freshwater species is typically initiated when semen is diluted in water (or in another hypotonic solution), by a decrease on the osmolarity of the extracellular medium or the K^+ ion concentration, and an increase in the concentration of free Ca^{++} ions (Morisawa et al. 1983). Urine or water contamination during semen collection can induce sperm motility and decrease the quality of frozen sperm. In *S. glanis*, it is a common practice to strip semen directly onto a hypertonic immobilizing NaCl-tris solution (~429 mOsm), as urine contamination is mostly inevitable (Linhart et al. 2005). A 244-mOsm saline extender (Ginsburg fish Ringer; Viveiros et al. 2000) and a 355-mOsm 6% fructose (Urbanyi et al. 1999) are suitable medium for freezing of *C. gariepinus* semen, while a 280-300-mOsmol/kg Hanks' balanced salt solution (HBSS) is extensively used as semen extender of *I. furcatus* (Bart et al. 1998, Lang et al. 2003), *I. punctatus* (Christensen and Tiersch 2005), *P. gigas* (Mongkonpunya et al. 2000a; Mongkonpunya et al. 2000b). Furthermore, several researchers have reported a pH effect on sperm motility. In numerous species, pH is involved in the control of flagellar movement and the optimal pH values seem to be species-specific according to the seminal plasma pH which has been reported as 7.7

in *C. gariepinus* (Horvath and Urbanyi 2000, Urbanyi et al. 1999), 8.0 in *M. nemurus* (Muchlisin et al. 2004), 8.3 in *P. hypophthalmus* (Mongkonpunya et al. 2000a) and 8.2 in *P. gigas* (Mongkonpunya et al. 2000a). As a consequence, most of the extenders suitable for freezing catfish semen are adjusted to a pH between 7.0 and 7.9 (Table 3).

Catfish semen has been diluted in freezing media at different ratios. Some researchers use a low 1:1 ratio (Horvath and Urbanyi 2000, Urbanyi et al. 1999), while others prefer a higher ratio of 1:10 (Viveiros et al. 2000, Viveiros et al. 2001) or even 1:20 (Muchlisin et al. 2004). Semen is mostly stored on LN_2 at -196 °C, and at this temperature cell viability can be held in a genetically stable form and is affected only by background radiation (Stoss 1983). The importance of preserving genetic resources for the future is widely recognized, and the conservation of semen would be a major contribution with great potential application in agriculture, biotechnology, species conservation, and clinical medicine. As males are killed for artificial reproduction in clariid and ictaluriid catfish culture, semen cryopreservation offers a unique tool for genetic preservation.

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Cryopreservation of Sperm of Asian Catfishes Including the Endangered Mekong Giant Catfish

Krit Mongkonpunya, Thusanee Pupipat and Terrence R. Tiersch

Introduction

In recent yr aquaculture has become an important sector of fish production in the region of the Mekong River. Catfishes of the families Pangasiidae and Clariidae are commercially produced throughout the region. Indigenous species include the Chao Phraya catfish, or swai, *Pangasius hypophthalmus* (formerly *P. sutchi*) (Roberts and Vidthayanon 1991), the Mekong giant catfish, or pla buk, *Pangasius gigas* (formerly *Pangasianodon gigas*), the walking catfish *Clarias batrachus*, and bighead catfish, *Clarias macrocephalus*. The Mekong giant catfish is the world's largest freshwater catfish, often surpassing 250 Kg in weight (Figure 1). This fish is of considerable popularity in Thailand, Laos, and Cambodia, and has been the object of a traditional capture fishery for centuries. Abruptly declining harvests have indicated that the Mekong giant catfish is an endangered species. The Thai Fisheries Department initiated an artificial breeding program for wild-caught Mekong giant catfish in 1983 and produced 200,000 fry which were reported to have attained an average of 21 Kg in 4 yr in earthen ponds (Pholprasith 1996). Appropriate aquaculture techniques have been developed for *P. gigas* and hybrids (*P. hypophthalmus* x *P. gigas*). Moreover, aquaculture production of commercial scale is in the expansion stage.

Figure 1. Transport of a 200-Kg Mekong giant catfish taken from the Mekong River in Northern Thailand (photograph by T. Tiersch).

Mongkonpunya, K., T. Pupipat and T. R. Tiersch. 2011. Cryopreservation of Sperm of Asian Catfishes Including the Endangered Mekong Giant Catfish. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 398-408.

For *C. batrachus* and *C. macrocephalus* commercial culture has largely ceased following the introduction of the faster growing hybrid of *C. macrocephalus* x the African catfish *C. gariepinus* which is available in markets and is grown on a commercial basis. In addition, pollution in the Mekong region is more serious than ever. Thus, the indigenous clariids are also threatened with extinction from natural waters. Cryopreservation of fish sperm is one possible means of assisting culture and conservation (Table 1).

Table 1. Applications of sperm cryopreservation in cultured and endangered fishes.

1. Long-term storage for regular or future use.
2. Genetic improvement through selective breeding programs.
3. Production of reference stocks for culture or research.
4. Production of hybrids.
5. Reduction of the cost and labor of maintaining broodstocks.
6. Elimination of the need for spawning "reproductive" synchronization of males and females.
7. Repeated spawning of specific males.
8. International shipment and use.
9. Germplasm conservation and development of germplasm repositories.
10. Use in conjunction with interspecific androgenesis to restore endangered or extinct species (e.g. Thongpan et al. 1997, Mongkonpunya et al. 1997).

Even though many species of shrimp and fish of the Mekong region are successfully cultured on a commercial basis, farming is not practiced with genetically improved seedstocks, and genetically improved catfish are virtually unavailable. The characteristics of an improved stock would include uniform size, fast growth, efficient food conversion, high dress-out percentage and disease resistance. Sperm preservation could be adopted for catfish culture in the region. With an effective sperm storage technique, catfish sperm could be transported to hatcheries to produce desirable hybrids or crosses. With adequate post-thaw motility and fertilization, cryopreserved sperm could provide hatcheries with improved seedstocks sufficient for commercial production.

We developed procedures for collection, handling, refrigerated storage (4 to 6 °C) and cryopreservation of sperm of pangasiid and clariid catfishes. Our objectives were to: 1) determine the physiochemical composition of the seminal plasma and blood serum; 2) examine the relationship of osmotic pressure and sperm activation to allow safe storage and to assure complete activation for fertilization; 3) evaluate the toxicity and effectiveness of different cryoprotectants, and 4) evaluate the success of different freezing rates. Motility estimates and tests of fertilizing capability were used to measure sperm viability. The techniques and equipment used in cryopreservation of sperm have been simplified to provide application or adaptation in other laboratories around the world.

Table 2. Inorganic electrolytes (molarity), osmolality (mOsmol/Kg) and pH of blood serum and seminal plasma of *Pangasius gigas* and *P. hypophthalmus*. Sample sizes are indicated in parentheses.

	P. gigas		*P. hypophthalmus*	
	Blood serum	Seminal plasma	Blood serum	Seminal plasma
mOsmol/Kg	232 ± 17 (8)	267 ± 45 (5)	273 ± 1 (15)	264 ± 3 (15)
PH	7.5 ± 0.3 (5)	8.2 ± 0.8 (5)	7.1 ± 0.5 (10)	8.3 ± 0.6 (10)
Ca^{++} (M)	3.5 ± 3.2 (3)	0.78 ± 1.1 (2)	ND (8)	ND (8)
K^{+} (M)	ND (1)*	ND (2)	0.01 ± 0.01 (16)	0.01 ± 0.01 (8)
Cl^{-} (M)	0.22 (1)	0.76 ± 10.4 (2)	0.03 ± 0.03 (5)	0.93 ± 0.54 (10)
Na^{+} (%)	1.52 (1)	0.43 ± 0.06 (2)	1.67 ± 0.56 (10)	1.51 ± 0.56 (6)

*ND, not detected; below detection level by the ion-selective and pH electrodes (Orion, Boston, Massachusetts).

Sperm Density and Seminal Plasma Physiochemistry

Undiluted pangasiid semen contained tremendous numbers of spermatozoa ranging from 2×10^{10} to 4×10^{10} sperm per mL in *P. gigas* (Mongkonpunya et al. 1996) to 7.6×10^{10} sperm per mL in *P. hypophthalmus* (Hambananda and Mongkonpunya 1996a). The mean pH of blood serum (~7.3) was lower than the pH of seminal plasma (~8.2) in each species (Table 2). The higher pH of seminal plasma could indicate contamination of stripped semen with urine (which tends to be alkaline). The mean osmotic pressure of blood serum and seminal plasma (230 to 270 mOsmol/Kg) were not significantly different between the two species or within species. Potassium ion concentration in blood serum and seminal fluid was not detectable (below the sensitivity limit) in *P. gigas* and it was 0.01 M in *P. hypophthalmus*. In contrast, calcium levels in the body fluids of *P. gigas* were relatively high (to ~ 3.5 M), but calcium was not detectable in blood serum of *P. hypophthalmus*. The major cation in the blood serum of each species was Na^{+} (0.4 to 1.8%) while Cl^{-} was the major anion. Results from these studies indicated that osmolality of any extender solutions should be adjusted to about 300 mOsmol/Kg to avoid sperm activation due to reduced osmotic pressure (Mongkonpunya et al. 1996). The effects of Ca^{++} and K^{+} on sperm activation should also be given careful consideration.

Extenders and Refrigerated Storage

Extenders are generally designed to be compatible with the physiochemical composition of the seminal plasma of the candidate species to maintain the sperm in a non-motile but viable state (Stoss 1983). Motility and fertilizing capability of pangasiid sperm diluted (1:3) in 0.9% normal saline (NaCl) or calcium-free Hanks' balanced salt solution (C-F HBSS) (Tiersch et al. 1994) (Table 3) could be maintained for 6 d at 5 °C with reduced motility (10 to 60%) and about 30 to 40% fertilization (Table 4) which was comparable to that of fresh sperm (~40 to 50% fertilization). A refrigerated sample of *P. gigas* sperm used in this study was also used by the Thai Fisheries Department for production of 50,000 *P. gigas* fry on d 7 of storage (~50%

motility) when a ripe female was available without a male or fresh sperm (1997 Technical Report, Inland Fisheries Section, Thai Fisheries Department).

Table 3. Ingredients (g/L) and osmolarity (mOsmol/Kg) of Hanks' balanced salt solution (HBSS), calcium-free Hanks' balanced salt solution (C-F HBSS), bicarbonate buffer (BCB) and 0.9% NaCl used to dilute sperm of pangasiid and clariid catfishes. Each mL of the extender also contained 500 IU of penicillin and 1 mg of streptomycin.

Ingredient	HBSS	C-F HBSS	BCB	NaCl
$CaCl_2 \cdot H_20$	0.16	--	--	--
NaCl	8.00	8.89	--	9.00
KCl	0.40	0.44	--	--
$MgSO_4 \cdot 7H_2O$	0.20	0.22	--	--
$Na_2HPO_4 \cdot 7H_2O$	0.12	0.13	--	--
KH_2PO_4	0.06	0.07	--	--
$NaHCO_3$	0.35	0.39	--	--
Glucose	1.00	1.11	--	--
$KHCO_3$	--	--	12.50	--
Sucrose	--	--	40.71	--
Glutathione, reduced	--	--	3.00	--
mOsmol/Kg	286	320	356	300

Table 4. Motility and fertilization observed for pangasiid sperm stored at 4 to 6 °C in bicarbonate buffer (BCB), calcium-free Hanks' balanced salt solution (C-F HBSS) or 0.9% sodium chloride (NaCl) for 6 d. Aliquots of sperm (~1 uL) were activated with 20 uL of distilled water.

	Percent motility during storage (hr)							
	0.5*	24	48	72	96	120	144	Percent fertilization**
Pangasius gigas								
BCB	100	10	0	--***	--	--	--	0
C-F HBSS	100	100	100	100	90	60	20	34 $\pm$ 8
NaCl	100	100	100	100	100	90	60	44 $\pm$ 17
Fresh sperm	--	--	--	--	--	--	--	47 $\pm$ 7
P. hypophthalmus								
BCB	40	--	--	0	--	--	--	0
C-F HBSS	80	--	--	30	--	--	10	10 $\pm$ 5
NaCl	80	--	--	20	--	--	10	27 $\pm$ 8
Fresh sperm	--	--	--	--	--	--	--	50 $\pm$ 3

* Motility was examined ~30 min after dilution at 1:3 (sperm:extender).
** In fertilization trials (on d 6) the eggs of *P. hypophthalmus* were used with a ratio of 4.2×10^6 sperm per egg.
*** Non-motile samples were discarded.

Because these extenders (NaCl and C-F HBSS) were significantly different in ionic composition when adjusted to 300 mOsmol/Kg, it was evident that maintaining osmotic pressure at levels isotonic or hypertonic to sperm cells was the main factor in maintaining viability and fertilizing capability of pangasiid sperm. Moreover, reduction in osmotic pressure played a major role in activation of sperm motility of *P. gigas* and *P. hypophthalmus* (Mongkonpunya et al. 1996). However, detrimental effects of bicarbonate buffer (BCB) on sperm motility were observed at 300 mOsmol/Kg. Sperm viability could not be extended longer than 24 hr even with dilution in glutathione-free BCB and replacement of $KHCO_3$ with Tris or the zwitterionic buffer HEPES (Hambananda and Mongkonpunya 1996a). Sperm of *P. gigas* diluted (1:3) in a mixture of 25% BCB and 75% 0.9% NaCl could be stored for 5 d with 60% motility, comparable to sperm stored in C-F HBSS, but with reduced motility (30%) compared to sperm stored in 0.9% NaCl alone (Table 5).

Table 5. Percent motility of *Pangasius gigas* sperm stored for 5 d at 5 °C in bicarbonate buffer (BCB), 0.9% sodium chloride (NaCl) or calcium-free Hanks' balanced salt solution (C-F HBSS) or mixture of BCB with the other extenders. The dilution ratio was 1:3 (sperm:extender). Aliquots of sperm (~1 µL) were activated with 20 µL of distilled water.

Extender mixture ratio	Osmotic pressure (mOsmol/Kg)	Percent motility during storage (hr)					
		0.5*	24	48	72	96	120
BCB:C-F HBSS							
1:0	356	100	10	0**	--	--	--
0:1	286	100	100	100	100	90	60
1:2	307	100	80	10	10	0	--
1:4	296	100	100	80	60	60	10
BCB:NaCl							
1:0	356	100	5	10	0	--	--
0:1	300	100	100	100	100	100	90
1:2	320	100	90	50	50	10	0
1:4	316	100	100	100	100	100	60
Undiluted sperm	300	100	100	20	20	0	--

* Motility was examined about 30 min after dilution.
** Non-motile samples were discarded.

Given our current knowledge of refrigerated storage of pangasiid sperm, use of saline solution (0.9% NaCl at 300 mOsmol/Kg) is recommended because it is inexpensive and easy to prepare. Moreover, pangasiid sperm diluted 1:1 either in 0.9% NaCl or C-F HBSS, held at half-volume (2.5 mL) in 5-mL cryotubes and stored on ice could be transported overnight (~ 12 hr) from the field to the laboratory for cryopreservation. Motility of the transported sperm was equal to the motility at the time of collection, allowing cryopreservation to be performed under controlled conditions. This is important for work with *P. gigas*, where the sperm is collected in the northern village of Cheng Kong which has no facilities for laboratory work. By transporting

the samples ~800 km by train to Bangkok, we could utilize a controlled-rate freezer and laboratory available at Kasetsart University.

Standardization and Cryopreservation Protocols

Utilization of information in many published protocols for cryopreservation of fish sperm is difficult because the reports are incomplete, and given that only positive results are usually published, the true variability of results remains unknown. Because of these difficulties, development of reliable cryopreservation protocols for fish sperm is often practiced on a species-by-species basis. To assist in sharing knowledge, emphasis should be placed on standardization of each step in a procedure. For example, sperm procurement, dilution ratios, extenders and cryoprotectants used, equilibration times, freezing and thawing rates, and egg-to-sperm ratios in fertilization tests can be standardized. Before any protocol is adopted, methods for evaluation should be carried out for each step.

Extenders and Cryopreservation

An inexpensive and easy method to prepare extender would be useful for the farmer, and it should be effective for refrigerated storage (described above) as well as for cryopreservation. Although HBSS of 280 to 300 mOsmol/Kg was an acceptable extender for sperm cryopreservation of channel catfish *Ictalurus punctatus* (Tiersch et al. 1994) and Mekong giant catfish (Mongkonpunya et al. 1995), we found that using 0.9% saline resulted in no difference in percent fertilization when compared to other extenders (Table 6).

Table 6. Fertilization based on number of eggs of *Clarias macrocephalus* inseminated with fresh semen of its own species (control) or with *Pangasius gigas* sperm cryopreserved in 10% dimethyl sulfoxide (DMSO) or propylene glycol (PG) in either 0.9% NaCl, bicarbonate buffer (BCB) or calcium-free Hanks' balanced salt solution (C-F HBSS) in 2-mL cryotubes and cooled at –10 °C per min using a controlled-rate freezer (Forma Scientific, New York). The cryopreserved sperm were thawed at 50 °C prior to use. The sperm to egg ratio was 4.2 x 10^6:1.

	Percent fertilization						
	10% DMSO			10% PG			
Female	NaCl	BCB	C-F HBSS	NaCl	BCB	C-F HBSS	Fresh sperm
1	84	66	70	65	67	78	74
2	19	18	41	15	45	23	23
3	4	7	2	7	3	2	5
4	8	9	3	7	2	1	27
Mean ± SD	29 ± 32	25 ± 24	29 ± 28	23 ± 24	29 ± 28	26 ± 31	27 ± 28

Toxicity of Cryoprotectants

Reductions in sperm motility and viability of pangasiid and clariid sperm were associated with increasing concentrations (5 to 14%) of dimethyl sulfoxide (DMSO), methanol or glycerol and increased exposure time (equilibration) before freezing (Mongkonpunya et al. 1995, Chairak and Mongkonpunya 1996). Among these chemicals, glycerol was more toxic to sperm than was DMSO, while methanol was the least toxic.

We used concentrations of 5 to 14% of each cryoprotectant, and held equilibration time at 15 min. Five percent DMSO in BCB or C-F HBSS was found to yield lower post-thaw motility than did 9% DMSO. However, at 14%, DMSO and methanol were too toxic for use in cryopreservation of pangasiid sperm, yielding 0% motility at 30 min equilibration (Mongkonpunya et al. 1995) (Table 7). Thus, to minimize the acute toxic effects, while maintaining the effectiveness of DMSO, we chose to use a concentration of 9% with a 15-min equilibration time as a general practice. Pre-freeze motility (at the start of the freezing process) of sperm exposed at this concentration was not different from the motility of sperm which were not exposed to the cryoprotectant.

Table 7. Percent motility of sperm of *Pangasius gigas* and *Clarias macrocephalus* stored at 5 °C for 30 min in extenders with various concentrations of cryoprotectants.

Species	Cryoprotectant in extender*	Percent motility	Reference
Clarias macrocephalus	8% glycerol in K-F CB	0	Chairak and Mongkonpunya 1996
	8% DMSO in K-F CB	43	Chairak and Mongkonpunya 1996
	8% Methanol in K-F CB	60	Chairak and Mongkonpunya 1996
Pangasius gigas	5% DMSO in C-F HBSS	100	Mongkonpunya et al. 1995
	5% MeOH in C-F HBSS	100	Mongkonpunya et al. 1995
	14% DMSO in C-F HBSS	0	Mongkonpunya et al. 1995
	14% MeOH in C-F HBSS	0	Mongkonpunya et al. 1995

*K-F CB, potassium-free modified Cortland's buffer; C-F HBSS, calcium-free Hanks' balanced salt solution.

Freezing and Thawing

Five or 10% DMSO, dimethyl acetamide (DMA) or glycerol, as well as 5% propylene glycol produced jelled samples with <1% post-thaw motility, while 10% propylene glycol yielded motility of <5%. We froze samples on aluminum canes in shipping dewars (Cryomed, Forma Scientific, Ohio). The average rate of freezing depended on the configuration of containers used (straws, tubes, etc.), the amount of liquid nitrogen (LN_2) and the position of the sample tubes on the canes. We found that the average rates of cooling were -40 °C per min for the lower position, -20 °C per min for the middle position, and -10 °C per min for the upper position. The post-thaw motility of sperm in the lower position was 0% for the four cryoprotectants studied at all concentrations. However, freezing in the middle and upper positions yielded ~5% motility after thawing.

In another study with Mekong giant catfish, sperm were frozen in LN_2 vapor with 8% DMSO in BCB using AI catheters (used for artificial insemination of cattle) as sperm containers. Prior to use, the sperm were thawed at 80 °C for 20 sec (Mongkonpunya et al. 1992). Fertilization percentages using eggs of *P. hypophthalmus* inseminated with cryopreserved (68%) and fresh sperm (79%) were not significantly different. In another study, *P. gigas* sperm in 5-mL cryotubes were frozen in LN_2 vapor with 9% DMSO in C-F HBSS or BCB at different freezing rates (Mongkonpunya et al. 1995). The cryopreserved sperm were thawed at 70 °C for 2 min prior to use. The sperm subjected to a cooling rate of -12 °C per min yielded the highest percent fertilization (66%), which was not significantly different from that of fresh sperm (74%). Although thawed sperm showed relatively high percent fertilization, few motile sperm were observed, whereas most sperm were motile in the untreated samples.

This might be surprising to those familiar with mammalian sperm where fertilizing capability is highly correlated with motility. From our experience, viability and fertilizing capability of pangasiid sperm were not directly correlated with motility. In many cases, sperm with no observable post-thaw motility yielded fertilization rates as high as those of fresh sperm with 80 to 100% motility. In fish, sperm penetration of the eggs is probably a function of the egg micropyle. Soon after the eggs are released from the female, both water and sperm could be drawn into the egg through the micropyle.

Overall, these studies represent more than a decade of research. Studies of *P. gigas* are especially difficult due to the high value and low numbers of fish captured, sometimes only allowing study of one or two males each yr. These studies have also been hindered by limited facilities at the collection sites, and recently by economic upheaval in Thailand. Despite these problems, workable techniques have been developed that are suitable for use with endangered species in developing countries.

Acknowledgments

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Annotated Bibliography of Developments in the Last Decade

The updated references (2000-2010) for this chapter include publications on biodiversity, culture, and cryopreservation for Asian catfishes, including *Pangasius hypophthalmus*, *P. sanitwongsei*, *P. larnaudii*, *P. bocourti*, *P. bjambal*, *Clarias macrocephalus*, *C. batrachus*, and *Mystus nemurus*. The references also include one review of 90 articles on minimizing genetic

adaptation in captive breeding of rare or endangered species, with recommendations including the integration of cryopreservation in minimizing genetic adaptations in the long-term preservation of genetic material and in maintaining biodiversity. For publicly available information on Mekong region ecology, management, fisheries, and aquaculture, pdf downloads can be obtained at *www.mrcmekong.org/free_download/research.htm#tab*.

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Cryopreservation Protocols for Sperm of Salmonid Fishes

Franz Lahnsteiner

Introduction

Simple, standardized and reproducible methods are necessary for routine application of semen cryopreservation in aquaculture. In the last yr we have developed a field cryopreservation method for semen of rainbow trout *Oncorhynchus mykiss* (Lahnsteiner et al. 1996a) and adapted it for lake trout *Salmo trutta f. lacustris*, brook trout *Salvelinus namaycush*, brown trout *Salmo trutta trutta*, Arctic charr *Salvelinus alpinus*, whitefish *Coregonus* sp. (Lahnsteiner et al. 1995, 1996a, 1996b), Danube salmon *Hucho hucho* and grayling *Thymallus thymallus* (Lahnsteiner et al. 1996c) and for an esocid species, the northern pike *Esox lucius* (Lahnsteiner et al. 1998a).

To ascertain reproducibility and to facilitate routine application of this cryopreservation technique, the optimal conditions and tolerable variations for the following parameters are summarized in this chapter: 1) storage period of semen before cryopreservation; 2) equilibration period in extender; 3) dilution ratios of semen in extender for efficient loading of straws; 4) freezing using liquid nitrogen (LN_2) vapor in an open system; 5) thawing to facilitate handling large numbers of straws; 6) the suitability of various straw sizes for different purposes (gene banking, insemination of large egg batches), and 7) biomarkers for predicting the suitability of semen for cryopreservation and the quality of thawed semen.

Methods

Materials

Mature 2 to 4 yr-old rainbow trout, lake trout, brook trout, brown trout and Danube salmon were obtained from Austrian fish farms. Arctic charr, whitefish and grayling were caught from local Austrian wild populations during spawning.

With the exception of female Danube salmon, the fishes were in spawning condition when used for the experiments. Spawning was induced in the female Danube salmon by intramuscular injection of freeze-dried carp hypophyses (4 mg/Kg body weight in 0.7% NaCl) 24 hr before egg collection. Semen was collected by gentle abdominal massage into vials, eggs were stripped into sieves and the ovarian fluid discarded. Sperm was processed according to the experimental design; egg batches from 3 to 6 females were pooled and used within 30 min for the experiments. Sperm density was measured using spectrophotometric methods (Ciereszko and Dabrowski 1993).

Cryopreservation Protocol

For cryopreservation experiments in each species, 4 to 8 semen samples were pooled in equal ratios to volumes of 5 to 20 mL according to the required amount. The semen was diluted in ice-cold extender (Preparation: dissolve 600 mg NaCl, 315 mg KCl, 15 mg $CaCl_2 \bullet 2H_2O$, 20 mg $MgSO_4 \bullet 7H_2O$, and 470 mg HEPES in 100 mL distilled water; adjust pH to 7.8; add 10 mL methanol, 1.5 g bovine serum albumin, 0.5 g sucrose and 7 mL hen egg yolk) at the desired ratio. In preliminary experiments a mixture of 5% dimethyl sulfoxide (DMSO) and 1% glycerol was used as cryoprotectant, but methanol proved to be superior (Lahnsteiner et al. 1997). The

Lahnsteiner, F. 2011. Cryopreservation Protocols for Sperm of Salmonid Fishes. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 409-420.

diluted semen was drawn into 0.5-mL, 1.2-mL or 5-mL straws. The unsealed straws were frozen in an insulated box in the vapor of LN_2 on a horizontally mounted tray (Figure 1). After a freezing period of 10 min (0.5-mL and 1.2-mL straws) or 15 min (5-mL straws) straws were placed in LN_2 for storage. For thawing, the straws were immersed in a thermostat-regulated water bath. Details on freezing and thawing conditions are described in the results. Immediately after thawing, the straws were removed from the water bath, opened and the semen poured onto the eggs.

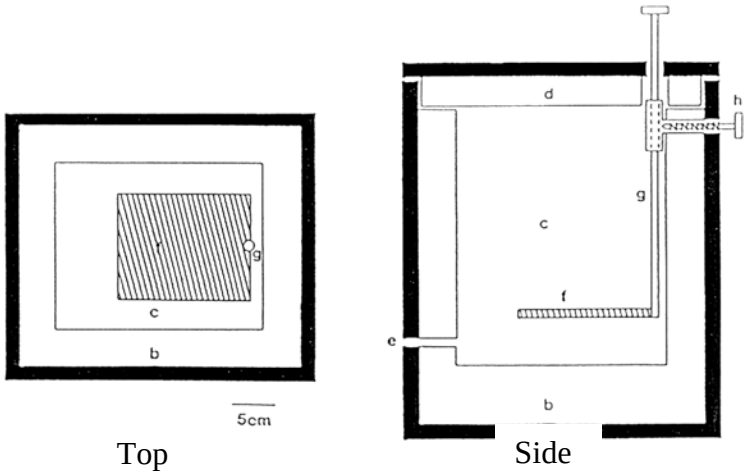

Figure 1. Insulated freezing box used for cryopreservation of salmonid semen. Scheme: a) exterior wall; b) insulating layer; c) freezing chamber; d) cover of freezing box; e) overflow trap for LN_2; f) platform for straws; g) attachment bar of platform; h) set screw.

Fertilization Assays

To exclude the influence of variability in gamete quality, the same semen and egg pools were used for related experiments. Fertilization with untreated (control) semen and thawed semen was performed in a similar way and at similar ratios of sperm to eggs. All fertilization trials were repeated at least 3 times.

In the fertilization assays, egg batches of 12.5 mL were transferred into Petri dishes about 2 min before fertilization and 6.25 mL of 4 °C fertilization solution was added (modified after Scheerer and Thorgaard 1989) (60 mM $NaHCO_3$, 20 mM glycine, 5 mM theophylline, 50 mM tris, pH 9). Other experiments revealed that glycine and theophylline did not affect post-thaw fertilization and could be omitted (Lahnsteiner et al. unpublished data). The desired amount of fresh or thawed semen was mixed with the eggs by gentle stirring. After 2 to 3 min the eggs were rinsed in well water and incubated in jars (whitefish) or flow-through incubators (Danube salmon, rainbow trout, lake trout, brook trout, brown trout, arctic charr and grayling). Fertilization rate was determined by the number of eyed stage embryos in relation to the total number of eggs.

Determination of Semen Quality

Semen from 50 mature rainbow trout was collected with catheters and processed (Figure 2) within 15 min after collection to define the suitability of semen for cryopreservation and the quality of cryopreserved semen.

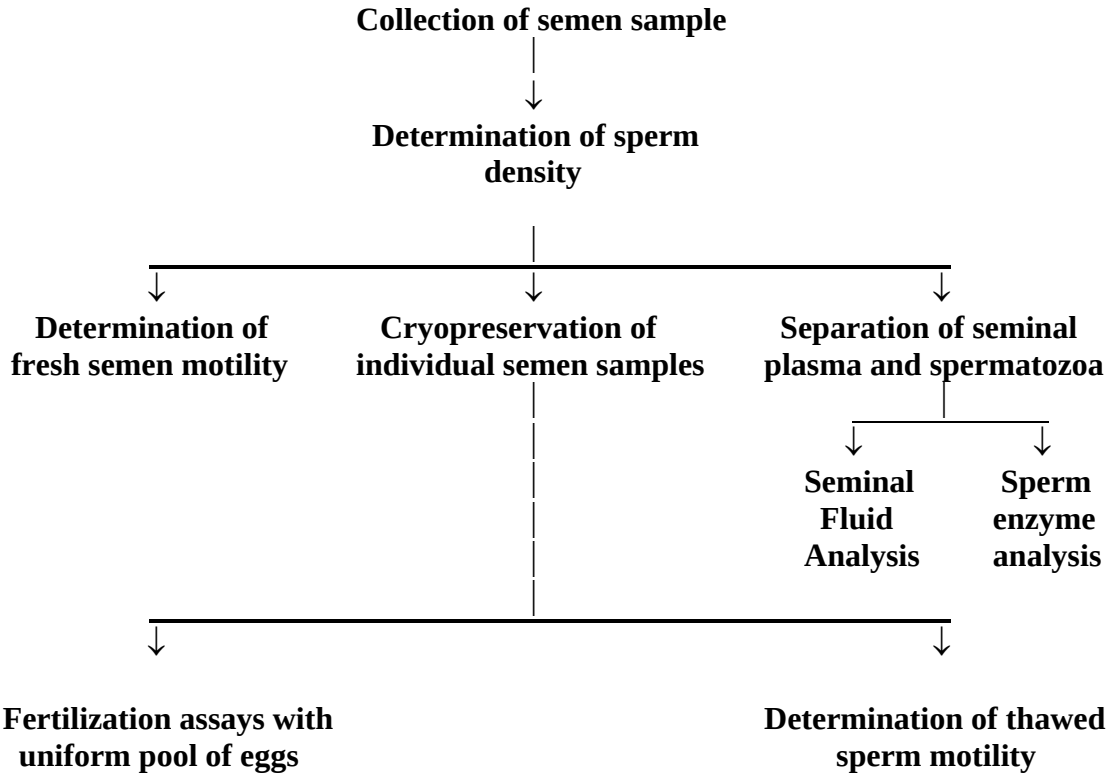

Figure 2. Processing of samples to determine the suitability of semen for cryopreservation and the quality of thawed semen.

Motility was determined with computer-assisted cell motility analysis (CMA) with an inverse phase-contrast microscope coupled with a video camera. Fresh semen samples were diluted 1:3 in buffered physiological saline (103 mM NaCl, 40 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM HEPES, pH 7.8). Thawed samples were diluted in the cryopreservation extender. Fertilization solution (20 µL) was placed into a specialized chamber for CMA analyses (20 µL volume, 10 µm depth), 1µL of diluted semen was added and the sample was vigorously mixed. The chamber was closed with a coverslip, transferred to the microscope and motility was recorded on videotape at 250-× magnification from a defined frame in the microscopic field. Motility was analysed on the video sections at 10 ± 1 sec after onset with the Stroemberg-Mika cell motility analysis program. The parameters are reported elsewhere (Lahnsteiner et al. 1996d).

Separation of seminal plasma and spermatozoa and extraction of spermatozoal enzymes followed routine procedures (Lahnsteiner et al. 1996d). The seminal fluid was analysed for osmolality, pH, Na^+, K^+, Ca^{++}, protein, cholesterol, choline, creatine phosphate, fructose, glucose, lactate, pyruvate and triglycerides and for the enzymatic activity of acid phosphatase, adenosine triphosphatase, alkaline phosphatase, aspartate aminotransferase, ß-D-glucuronidase and lactate dehydrogenase. In spermatozoa, the activities of acid phosphatase, adenylate kinase,

adenosine triphosphatase, ß-D-glucuronidase, isocitrate dehydrogenase, lactate dehydrogenase, malate dehydrogenase and pyruvate kinase were assayed. All analysis procedures followed published methods (Lahnsteiner et al. 1996d).

Statistical Analysis

Percentage data were subjected to angular transformation. Student t-test or one-way analysis of variance with Newman Keul's test was used to compare the mean values at a probability level of $P < 0.001$. Metabolite values and enzyme activities of the seminal fluid were standardized to a defined volume of seminal fluid; those extracted from spermatozoa were standardized to the protein content of the crude enzyme extract. Pearson correlation coefficients were used for metrical data, regression analyses were done with linear and non-linear regression models.

Results and Discussion

Storage of Untreated Semen Before Cryopreservation

Subsamples of three different semen pools of rainbow trout were cryopreserved immediately after collection (<10 min) and after storage periods of 1 and 2 hr. For storage, 1-mL portions of semen were placed in vessels at a depth of 1 cm without further treatment, a procedure which is common in routine semen handling. This allowed easy sampling, and avoided the loss of semen on the surface of wide-bottom flasks which are necessary for thin layer storage under aerobic conditions (Billard 1981, Stoss and Holtz 1983a). For semen cryopreserved within 10 min after collection, and for semen stored for as long as 60 min before cryopreservation, the post-thaw fertilization rates were about 60 to 80% (pool I: 60 ± 11%, pool II: 82 ± 3%, pool III: 61 ± 6%; fresh semen control: pool I: 62 ± 11%, pool II: 88 ± 1%, pool III: 83 ± 8%) using a ratio of 1 x 10^6 to 6 x 10^6 spermatozoa per egg. For semen stored 120 min before cryopreservation, the fertilization capacity was significantly reduced to about 40 to 60% (pool I: 47 ± 17%, pool II: 33 ± 1%, pool III: 38 ± 8%).

After a storage period of 2 hr, the fertilization capacity of fresh semen was reduced by about 35%, the motility was reduced by 65% and swimming velocity was reduced by 20% (Lahnsteiner et al. 1998b). Other changes concerned the composition of the seminal fluid and sperm metabolism (Lahnsteiner et al. 1998b). These data clearly indicated that storage of semen for periods longer than 1 hr induced physiological and metabolic changes and decreased semen quality. As a practical recommendation, storage of untreated semen before cryopreservation should not exceed 1 hr, a time span sufficient for the initial handling steps of collection, quality control, dilution and freezing.

Dilution Ratio of Semen in the Extender

To establish efficient loading of straws, the dilution ratio of semen in extender must be minimized. Tolerable minimal dilution ratios were species-specific. In Arctic charr, dilution ratios of semen in the extender of less than two-fold decreased significantly the post-thaw fertilization rate (dilution ratio of 1:2 yielded 65 ± 4%, 1:1 yielded 52 ± 2%, fresh semen control yielded 70 ± 4%) using a ratio of 4.2 x 10^6 spermatozoa per egg. In Danube salmon, rainbow trout, brook trout and grayling, dilution ratios of less than three-fold decreased fertilization rates (e.g. rainbow trout: dilution ratio of 1:3 yielded 82 ± 3%, 1:1 yielded 62 ± 4%, fresh semen control yielded 88 ± 1%) at a ratio of 5.0 x 10^6 spermatozoa per egg. In brown trout, dilution

ratios of less than five-fold decreased fertilization rates (dilution ratio of 1:7 yielded 86 ± 2%, 1:5 yielded 77 ± 3%, fresh semen control yielded 92 ± 1%;) at a ratio of 5 x 10^6 spermatozoa per egg. In lake trout, dilution ratios of less than seven-fold decreased fertilization rates (dilution ratio of 1:7 yielded 73 ± 1%, 1:5 yielded 65 ± 3%, fresh semen control yielded 80 ± 4%) using a ratio of 9 x 10^6 spermatozoa per egg. Also sperm density was species-specific. For the semen pools it amounted to 0.8 x 10^9 to 1.1 x 10^9 cells per mL for Arctic charr, 3.2 x 10^9 to 6.5 x 10^9 cells per mL for Danube salmon, rainbow trout, brook trout and grayling, 7.0 x 10^9 to 8.1 x 10^9 cells per mL for brown trout and 1.8 x 10^{10} to 2.3 x 10^{10} cells per mL for lake trout.

These results lead to the conclusion that minimal dilution ratios of semen in the extender depended on sperm density, and sperm concentration should not be higher than 2.0 to 2.5 x 10^9 cells per mL of diluent. At higher cell concentrations the post-thaw fertility of semen significantly decreased probably due to damage because of limited intercellular space and cell compression. Decreasing the sperm concentration to 7.6 x 10^8 spermatozoa per mL of extender did not significantly influence the fertilization rate (Lahnsteiner et al. 1996b), but lead to inefficient loading of straws. This is a disadvantage, especially when numerous straws have to be handled.

For practical application, we recommended a two-fold dilution of semen in extender for Arctic charr, a three-fold dilution for whitefish, Danube salmon, rainbow trout, brook trout, grayling, a five-fold dilution for lake trout and a seven-fold dilution for brown trout.

Influence of Equilibration Period on the Post-thaw Fertility

For salmonid spermatozoa, long equilibration periods in the extenders are not necessary as the cells are small (maximal width of head 2 to 3 µm) (Lahnsteiner et al. 1992), and have no permeability barriers that limit cryoprotectant penetration. Further, extender compounds may be toxic to sperm cells and therefore equilibration should be kept as short as possible (freezing within 1 min after dilution) (Legendre and Billard 1980, Stoss and Holtz 1983b, Piironen 1993).

Experiments with rainbow trout demonstrated that for spermatozoa (diluted in the extender described here), equilibration periods of 1 min were sufficient (post-thaw fertilization of 84 ± 5%, fresh semen control 88 ± 1% at a ratio of 5 x 10^6 spermatozoa per egg) and post-thaw fertilization was not improved by a 3-min or a 5-min equilibration (82 ± 3%). However, equilibration periods of 10 min and 20 min did not significantly affect the post-thaw fertility of semen. Similar results were obtained for whitefish, Danube salmon, brook trout, grayling, lake trout and brown trout (Lahnsteiner unpublished data). Therefore, the extender had no negative effects on spermatozoal physiology and metabolism and the tolerance of spermatozoa to equilibration periods of as long as 20 min facilitated the dilution, loading in straws and freezing of large semen volumes. This is an important advantage for cryopreservation techniques.

Suitability of 0.5-mL, 1.2-mL and 5-mL Straws for Cryopreservation of Salmonid Semen

Straws with volumes of 0.5 mL or 1.2 mL were suitable for cryopreservation of salmonid semen when freezing and thawing conditions were adapted depending on straw size and species (Figures 3 and 4). The conditions for straws for Danube salmon, rainbow trout, brown trout, lake trout and grayling were: 0.5-mL straws frozen at 1.5 cm (-110 ± 2 °C) above the level of LN_2 and thawed at 25 °C for 30 sec; 1.2-mL straws frozen at 1.0 cm (-130 ± 2 °C) above LN_2 and thawed at 30 °C for 30 sec. The conditions for straws for brook trout and Arctic charr were: 0.5-mL straws frozen at 2.5 cm (-92 ± 2 °C) above the level of LN_2 and thawed at 25 °C for 30 sec; 1.2-mL straws frozen at 2.0 cm (-100 ± 2) above the level of LN_2 and thawed at 30 °C for 30

sec. Freezing semen in 5-mL straws at 0 to 1 cm above the level of LN_2 and thawing at 35 to 40 °C yielded only 39% of the post-thaw fertilization rate obtained with 0.5-mL and 1.2-mL straws.

The rates were similar when freezing in either 0.5-mL straws at -110 ± 2 °C or 1.2-mL straws at -130 ± 2 °C and in 0.5-mL straws at -92 ± 2 °C or 1.2-mL straws at -100 ± 2 °C (Lahnsteiner et al. 1997). Also, thawing rates were similar when thawing 0.5-mL straws at 25 °C for 30 sec or 1.2-mL straws at 30 °C for 30 sec (Lahnsteiner et al. 1997). However, in 5-mL straws the freezing and thawing rates were heterogeneous and slow and could not be optimized with the equipment used.

Straws with a volume of 0.5 mL are suited for gene banking and for freezing of small amounts of semen, straws with a volume of 1.2 mL have advantages for the fertilization of large egg batches. Wheeler and Thorgaard (1991) tested 5-mL straws for cryopreservation of rainbow trout semen and obtained fertilization success of about 50%. Straws of larger volumes would be better suited for large-scale insemination in aquaculture, and adequate straws and freezing and thawing conditions will have to be developed in the future.

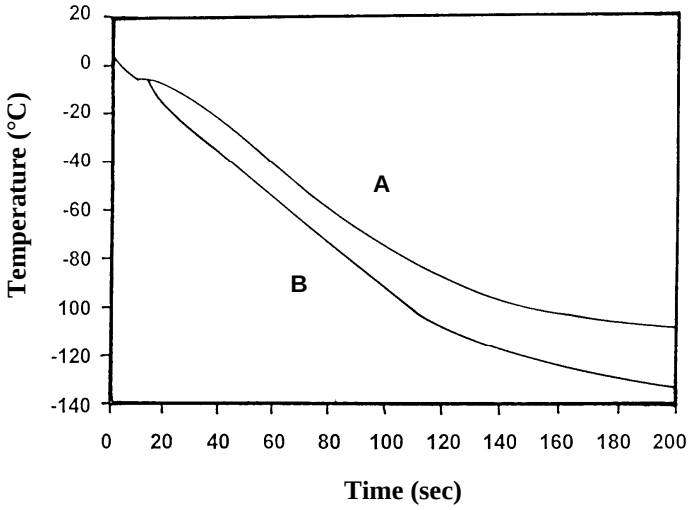

Figure 3. Optimal freezing curves for salmonid semen: A) brook trout, Arctic charr, B) Danube salmon, rainbow trout, brown trout, lake trout and grayling. Determinations were based on eight independent measurements in 1.2-mL straws and represent mean values; the standard deviations were ≤ ± 2 °C.

Tolerance to Deviations from the Optimal Freezing Conditions

For semen of all species investigated and for 0.5-mL and 1.2-mL straws, a deviation of 0.5 cm from the optimal freezing level resulted in a significant decrease of post-thaw fertilization rate. For example, for rainbow trout semen loaded in 0.5-mL straws the post-thaw fertilization was 84 ± 7% at the optimal freezing level of 1.5 cm (-110 °C) above the level of LN_2, but was significantly reduced to 63 ± 10% at 1 cm (-130 °C) above the level of LN_2 and to 63 ± 12% at 2 cm (-100 °C) above the level of LN_2. The fresh semen control yielded 85 ± 6% fertilization at a ratio of 3.5×10^6 spermatozoa per egg. Thus it is important in practice to maintain the freezing temperature by adjusting the freezing levels due to continuous LN_2 evaporation.

Tolerance to Deviations from the Optimal and Thawing Conditions

Thawing is the most sensitive parameter in salmonid semen cryopreservation and slight deviations from optimal conditions can lead to a significant reduction of fertilization success (Lahnsteiner et al. 1995, 1997). For example, the post-thaw fertilization rate of semen of grayling loaded in 0.5-mL straws was 89 ± 5% when thawed at 25 °C for 30 sec (fresh semen control 88 ± 1% at a ratio of 1.6×10^6 spermatozoa per egg). When thawing was shortened to 25 sec, the post-thaw fertilization rate was reduced significantly to 68 ± 8%. When thawing was prolonged to 35 sec, the post-thaw fertilization rate was reduced to 67 ± 1%. Changes in the thawing temperature

also resulted in a significant reduction of post-thaw fertilization rates. The post-thaw fertilization rate of semen at 30 °C for 25 sec was 68 ± 7%, while the post-thaw fertilization rate at 15 °C for 15 sec was 71 ± 2%. Using the optimal thawing conditions, semen was warmed to 20 °C (Figure 4), a temperature higher than the physiological optimum (Lahnsteiner et al. 1995). We suggest that either recrystalization (the formation of ice crystals during thawing) is reduced or avoided by this thawing procedure or enzymatic activities are maintained. The sensitivity of semen to changes in thawing conditions complicates the insemination of large egg batches because numerous straws have to be processed under exactly constant conditions.

There is no possibility to store thawed semen before fertilization. In rainbow trout, the post-thaw fertility of semen used for fertilization immediately after thawing was 84 ± 7% (fresh semen control 85 ± 6% at a ratio of 3.5 x 10^6 spermatozoa per egg). But when the thawed semen was kept in the straw for 30 sec in air at 8 to 10 °C before fertilization, the fertilization rate was reduced significantly to 58 ± 21%. This rapid decrease in semen quality might be due to cell injuries such as swelling. Rupture of the mitochondria and the plasmalemma are frequently observed by electron and light microscopical analysis of thawed semen (Lahnsteiner et al. 1996e).

Sperm-to-egg Ratio

To obtain fertilization rates of 90% to 100% of the fresh semen control, reliable insemination doses with thawed semen were 20 mL of diluted thawed semen per egg batch of 500 mL for all species investigated. Because species-specific differences in sperm density were compensated for by the dilution ratio of semen, this amount represents about 4 to 7 x 10^{10} spermatozoa per 500 mL of eggs. This resulted in a ratio of 2.5 to 5.5 x 10^6 spermatozoa per egg for Danube salmon, rainbow trout, Arctic charr, brook trout, lake trout and brown trout (in which 500 mL of roe yields 5,000 to 7,000 eggs). This also yields a ratio of 1.2 to 1.6 x 10^6 spermatozoa per egg for grayling *(*in which 500 mL of roe yields 20,000 eggs) and 3.0 to 5.0 x 10^5 spermatozoa per egg for whitefish (in which 500 mL of roe yields 75,000 eggs). The reason why lower sperm-to-egg ratios are necessary for whitefish and grayling than for the other species is unknown, as semen quality after cryopreservation revealed no species-specific differences. The amounts of cryopreserved semen used for fertilization were about 5 to 10 times higher than the amounts of fresh semen used for artificial insemination because thawed semen has significant alterations in morphology (damage of spermatozoa), physiology (decrease of motility) and biochemistry (decrease in enzyme activities and metabolite levels) (Lahnsteiner et al. 1996e).

Suitability of Fresh Semen for Cryopreservation and Quality of Thawed Semen

In the 50 samples of rainbow trout semen studied, post-thaw fertilization (54 ± 38%, mean ± standard deviation) was significantly correlated ($P < 0.01$) with the fresh semen motility rate (50 ± 5%, $y = 4.996x - 0.0958x^2 + 0.0006x^3 - 51.7363$) and with the fresh semen total average path swimming velocity (88 ± 15 µm per sec, $y = 6.741x - 0.036 x^2 - 268.37$). The post-thaw fertilization rate was also significantly correlated ($P < 0.01$) with the motility rate of thawed semen (8 ± 7%, $y = 1.943x + 28.002$) and the thawed semen total average path velocity (90 ± 23 µm per sec, $y = 0.812x - 0.0059x^2 + 24.968$). Therefore, in fresh semen a motility rate of > 80% and swimming velocities of between 80 and 100 µm per sec indicated good suitability for cryopreservation (post-thaw fertility of > 50%); in thawed semen, a motility rate > 20% and a swimming velocity of 60 to 80 µm per sec indicated high quality. Within the Salmonidae, motility parameters revealed no species-specific differences, neither in fresh nor in thawed

semen. Therefore, the regression models can also be applied for semen of Danube salmon, brown trout, lake trout, Arctic charr, brook trout and grayling. Semen quality determination by motility parameters is a reliable, quick and simple method especially when using cell motility analysis equipment.

From the biochemical parameters investigated, the post-thaw fertility of semen revealed significant correlations with pH (8.3 ± 0.2, y = -82.768x + 728.133), osmolality (313 ± 22 mOsmol/Kg, y = 0.539x - 125.59), triglyceride levels (201 ± 108 µM/L, y = 0.069x + 29.863), ß-D-glucuronidase activity (0.8 ± 0.6 µM/L per min, y = $-1.112x + 0.0058x^2 + 82.229$), lactate dehydrogenase activity (207 ± 78 µM/L per min, y = $-0.096x + 0.00006x^2 + 583.80$), spermatozoan acid phosphatase activity (0.3 ± 0.1 µM per min/100 mg protein, y = $-132.51x + 126.38x^2 + 66.48$), and spermatozoan adenylate kinase activity (9.6 ± 5.8 µM per min/100 mg protein, y = 3.474x + 4.925). Osmolality and pH were the parameters most easily determined and therefore are important for application in aquaculture. High osmolality (> 330 mOsmol/Kg) and a pH of less than 8.2 characterized semen with more than 50% post-thaw fertility. The distinct meanings of the described parameters for semen biology are discussed elsewhere (Lahnsteiner et al. 1996d, 1998c).

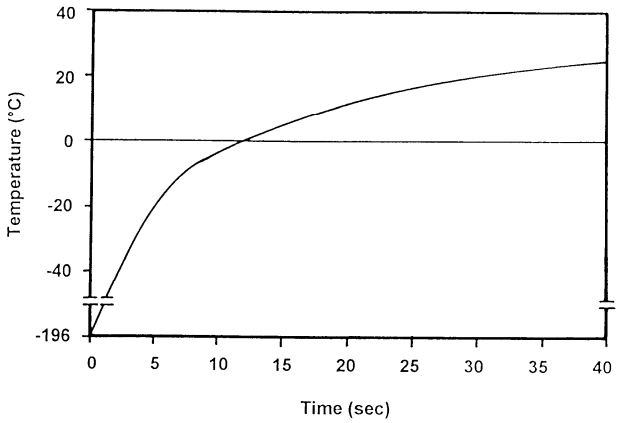

Figure 4. Optimal thawing curve for semen of Danube salmon, rainbow trout, brown trout, lake trout, brook trout, Arctic charr, and grayling. Determinations were based on eight independent measurements and represent mean values; the standard deviations were ≤ ± 2 °C.

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Annotated Bibliography of Developments in the Last Decade

Salmonids have remained an active area of research since the publication of this book in 2000, with sperm cryopreservation moving towards commercialization. Research has improved sperm storage of these fishes, but there are still aspects that need attention.

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Cryopreservation of Sperm of Steelhead Rainbow Trout after Refrigerated Storage

Joseph G. Cloud

Introduction

Genetic conservation of existing fish stocks is an important goal in itself and as a component of programs designed to insure a viable and sustainable fishery under changing environmental conditions. With the constant threat of losing genetic diversity in specific native fish stocks, the establishment of a program for the long-term storage of fish germplasm would serve as insurance for ongoing conservation programs.

At present, the cryopreservation of sperm is the only functional means of storing fish germplasm for extended periods of time. The successful cryopreservation of spermatozoa from the wide variety of marine and freshwater fish species documented in this book demonstrates that this is a functional procedure that can be utilized directly or modified to preserve the spermatozoa of all fish populations. In addition, the establishment of germplasm repositories, for extended storage of fish spermatozoa can also be utilized as a component of programs producing disease-free stocks and in breeding programs in which the spawning of males and females is not synchronized.

Because the freezing and thawing processes associated with the cryopreservation of cells are potentially detrimental, conditions that contribute to the maintenance of sperm fertility are important to identify. The capture of male fish does not always occur near facilities that can support sperm freezing. Therefore, there is the question of whether it is practical to establish a facility near the area that the fish are being trapped, or should the milt be transported to a more suitable location. The objective of this experiment was to determine if the fertility of thawed milt varied with the time of cryopreservation relative to the time of collection. The hypothesis of this experiment was that semen frozen on the d of collection would have a higher fertility than would semen frozen 24 hr after collection.

Methods

Sperm Collection

Milt was collected from ripe steelhead rainbow trout *Oncorhynchus mykiss* during the spring of 1997 by manual stripping and placed in individual, pre-cooled (4 °C) Whirl-pac® bags. Milt from six males was collected on April 15; milt from five males was collected on April 22, and milt from five males was collected on April 29. The milt was transported to the laboratory (1.5 hr) in a cooler at 2 to 4 °C. Within 4 hr of collection, an aliquot of the milt was cryopreserved. The remaining milt was stored at 2 to 4 °C for 24 hr in Whirl-pac® bags filled with oxygen before cryopreservation.

Cryopreservation of Milt

The milt was diluted (1:3) with a freezing solution consisting of a 300 mM glucose solution containing 10% dimethyl sulfoxide (Sigma Chemical Company, St. Louis**,** Missouri) and 13.3% egg yolk at 4 °C. The extended milt was immediately drawn into 0.5-mL straws. The

Cloud, J. G. 2011. Cryopreservation of Sperm of Steelhead Rainbow Trout after Refrigerated Storage. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 421-424.

straws were sealed, cooled at -30 °C per min in liquid nitrogen (LN_2) vapor to -70 °C and plunged into LN_2 for storage.

Post-thaw Fertility of Cryopreserved Milt

To evaluate the fertility of the frozen sperm, milt from all males in all treatment groups were thawed on the same d (May 6) and were used to fertilize the eggs from a single female (eggs were collected on the morning of May 6 and transported to the laboratory in a sealed plastic box at 4 °C). The straws were thawed in a water bath at ~12 °C. The milt was added to the eggs just as the thawing milt was going through the transition from solid to liquid. Each straw (which contained 125 μL of milt) was added to ~150 eggs. The eggs were incubated in individual containers within a Heath incubator at 11.5 to 12 °C. On d 9 of incubation, the eggs were fixed by placing them in a solution of 1.5% acetic acid in 0.9% saline. Fertilization was estimated from the percentage of embryos that were developing (had an embryonic kneel) at that time.

Statistical analysis

Differences in fertilization rates relative to the time of freezing were tested by use of a paired *t*-test. The null hypothesis was rejected for tests of significance when $P \leq 0.05$.

Results and Discussion

The average fertility of steelhead semen cryopreserved on the d of collection (34 ± 14%, mean ± SD) was not significantly different from the fertility of semen from the same males that was stored at 4 °C for 24 hr before cryopreservation (51 ± 14%). Previously, Stoss and Holtz (1983) demonstrated that salmonid milt held at cold temperatures would maintain fertility for a number of d.

These data support the conclusion that the fertility of sperm cryopreserved following 24 hr of storage is not different from that of sperm frozen on the d of collection. This conclusion leads to the recommendation that milt collected at remote sites be shipped to a location for cryopreservation. It must be stressed however that this is based on the assumption that the milt is carefully collected and that it is cooled, transported and stored properly. This finding is of practical value because it is generally less expensive to transport collected milt from remote regions or even from a hatchery than it is to transport the equipment and supplies for cryopreservation to a collection site. Additionally, maintaining quality control for sperm cryopreservation is more manageable in an established laboratory than at a busy hatchery or streamside.

Summary

Semen was collected from 17 steelhead trout during a 3-wk period, transported to the laboratory, cryopreserved on the d of collection and on the d following collection, and stored in LN_2. One week following the collection of the last milt samples, the frozen semen from all males was thawed and used to fertilize the eggs from a single female. The proportion of embryos developing normally from sperm frozen on the d of collection was not greater than the proportion of embryos derived from sperm frozen 24 hr later. These data do not support the original hypothesis. Under the conditions of the investigation, sperm collected and stored for 24

hr before cryopreservation have the same potential fertility as sperm frozen on the d of collection.

Acknowledgments

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There has been a moderate number of publications specifically related to rainbow trout cryopreservation since the first edition was published. In 1978, 1980 and 1984, three reviews of storage of salmonid sperm were published. After that, another review covered new methods and improvements in 2000. Research improving cryopreservation in these fish is continuing, but there are still many aspects that need attention.

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Cryopreservation of Sperm of the Endangered Apache Trout

Robert E. David, Lawrence J. Wirtanen and Michael A. Ternes

Introduction

Apache trout *Oncorhynchus apache* historically inhabited headwaters of the Salt and Little Colorado Rivers in the White Mountains of east-central Arizona. This fish has been noted in literature since the late 1800's, but it was not until 1972 that it was described as a distinct species (Miller 1972). The appearance of the Apache trout is unique among most North American salmonids. The overall gold coloration contrasts with normally scarce spots that are frequently outlined with a distinct pale halo. A yellow "cutthroat" slash is also present in the gular folds; however, the ancestry of this species is linked more closely to the rainbow trout *O. mykiss* than to that of the cutthroat trout *O. clarki* (Behnke 1992).

The Apache trout was listed as threatened under the Endangered Species Act of 1973. Recovery plans included provisions for artificial propagation of the species to assist in restoration efforts. Initial attempts to culture Apache trout were abandoned by the State of Arizona in 1981 and reinstated by the U.S. Fish and Wildlife Service in 1983. Production of Apache trout at the Williams Creek Unit of Alchesay-Williams Creek National Fish Hatchery (NFH) complex steadily increased from 1,200 fish in 1986 to over 500,000 fish in 1990. Currently (2000), the program produces 1.2 million eggs annually for use in several Federal and State propagation regimes for restoration and sports fishing enhancement. Varied success of reintroduction programs raised questions about the genetic ability of stock from a single isolated population to adapt to diverse habitats. While biochemical systematics proved captive brookstock to be "pure," it also revealed very little genetic variability within the population (Carmichael et al. 1993).

In order to enhance diversity within captive stocks, a study was initiated to test feasibility in the transfer of genetic material from wild to captive populations by field collection and use of male gametes. Spawning times of captive Apache trout precede those of wild populations by as much as 4 mo, prescribing the use of long-term storage techniques.

Methods

Cryopreservation trials were performed at the Williams Creek NFH in March and April of 1993. Remote streams containing pure populations of Apache trout were monitored in early spring to measure diurnal water temperature increases. Apache trout spawn in response to decreasing stream flow and rising temperatures, with a thermal trigger of about 7 °C. Attempts were made to collect milt samples from 20 to 50 fertile males from each stream depending on existing population sizes. Apache trout were randomly collected using backpack electrofishing equipment from all inhabited stream reaches. All males were palpitated in the field to confirm spermatogenesis and were temporarily held in net livecars until sufficient numbers were collected.

David, R. E., L. J. Wirtanen and M. A. Ternes. 2011. Cryopreservation of Sperm of the Endangered Apache Trout. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 425-429.

Males were anesthetized to initial loss of equilibrium in a 75 mg/L solution of tricaine methane sulfonate (MS-222), and were rinsed in stream water and blotted dry with a moist cotton towel. Sperm collection involved two people, one to hold the donor fish inverted and a sec to massage the abdomen and aspirate milt. The milt aspiration device (Figure 1) was designed with an ice bath jacket surrounding a 14-mL polystyrene collection tube to maintain collected milt at 1 to 2 °C. As wild males produce only a small volume of milt (< 0.5 mL), at least five males were pooled to avoid temperature shock and desiccation associated with handling of small milt samples. Following aspiration, contents of the collection tube were transferred to a 180-mL Whirl-pak®, inflated with pure oxygen and maintained at 1 to 2°C on crushed ice.

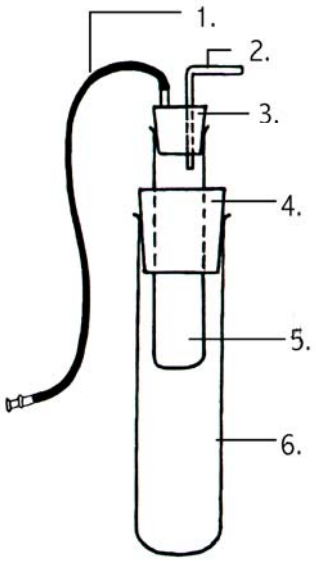

Figure 1. Salmonid milt aspirator: 1) 0.3 cm ID × 0.5 cm OD latex tubing; 2) 2.2mm ID × 3.2 mm polyethylene tubing; 3) two-hole "O" stopper; 4) # 6 ½ stopper bored with a # 6 borer; 5) 14-mL graduated Falcon tube; 6) polycarbonate round-bottom centrifuge tube (100 mL, 31.5 mm × 166 mm).

Cryopreservation

Milt samples were returned to the laboratory, maintained at 1 to 2 °C on ice, and were cryopreserved within 24 hr of collection. Cryopreservation methods were adapted from Cloud et al. (1990) with minor in-house modifications of technique and equipment. Milt was diluted volumetrically at a 1:1 ratio using a cryoprotectant solution of 300 mM glucose, adjusted to a pH of 6.0 using dilute HCl, 10% egg yolk and 7.5% dimethyl sulfoxide prepared as a 30-mL aliquot. Measurement of yolk volume was simplified by cracking a chicken egg into a shallow media dish lined with absorbent toweling. A dissecting needle was used to tear the yolk membrane and a 3-mL syringe (without needle) was inserted into the yolk at a depth of ~1 cm to measure and transfer without contamination. Mixing of the yolk was simplified by use of platform vortex mixer. Cryoprotectant, contained in a 50-mL glass Erlenmeyer flask, was submerged in an ice

bath and allowed to cool to 1 to 2 °C. A clean 50-mL glass Erlenmeyer flask was also pre-cooled in the same bath. Milt, held in the 180-mL Whirl-pak®, was measured and transferred to the clean flask using a pre-cooled plastic syringe and was diluted 1:1 with cryoprotectant and briefly mixed by swirling. Milt was aspirated into pre-cooled 0.5-mL cotton-plugged French straws, allowing a 10-mm air space and were sealed with Critoseal®. Filled straws were laid horizontally on a block of dry ice and covered with a thin sheet of polyethylene foam for 15 min following addition of the last straw.

Following initial freezing, straws were rolled off the dry ice and allowed to plunge into a liquid nitrogen (LN_2) bath. Polyethylene goblets (13 mm x 120 mm) mounted on aluminum canes were pre-cooled in a LN_2 dewar. Straws were transferred from the LN_2 bath to the goblets for storage at 7 straws per goblet, allowing sufficient space for easy handling of individual straws.

Spawning

Samples of 400 eggs were collected from Apache trout broodstock and about 50% of the ovarian fluid was removed to reduce dilution of milt during fertilization. Eggs were transferred to a 180-mL Whirl-pak®, inflated with pure oxygen and temporarily stored at 1 to 2 °C on crushed ice. Prior to fertilization, eggs were transferred to a 400-mL plastic bowl and tempered to 12 °C. A solution of 119 mM sodium chloride, adjusted to pH 9.0, was added to the eggs as a milt activator. Straws containing cryopreserved milt were removed from goblets and thawed by swirling in a 1000-mL beaker containing water and crushed ice at a temperature of 1 to 2 °C for 55 sec. The straws were held over eggs and the plugs clipped to allow the thawed milt to flow into the mixing container. Fertilization was enhanced by agitation for 15 to 20 sec. Eggs were held motionless for 45 to 60 sec, and were rinsed and transferred to compartments within Heath incubators for water hardening and incubation.

Results and Discussion

The first trial, using milt from a wild Apache trout population in Crooked Creek and eggs from hatchery stock, yielded fertility rates ranging from 1% to 5% with an average of 2%. The sec trial, using milt from wild Apache trout in Mamie Creek, yielded fertility rates ranging from 4% to 25% with an average of 12%.

Although fertility rates appeared low, sufficient quantities of wild and captive gametes resulted in the production of a significant number of offspring with a 50% wild genotype. These trout will be reared to maturity and used to selectively replace current broodstock. Trials indicate that the use of cryopreservation as a technique for long-term storage of Apache trout milt may prove to be a valuable tool in sustaining diversity in captive Apache trout broodstock through the infusion of wild genetic material. The maintenance of a polymorphic gene pool will help to ensure that Apache trout produced by artificial propagation and restored to reclaimed stream habitats have sufficient genetic integrity to adapt to these environments and produce a self-sustaining population, thereby contributing to efforts to remove this fish from the endangered species list.

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Much work has been done since 2000 in the area of cryopreservation of trout sperm. Studies have begun to focus on the effects of cryopreservation on genetics and function of sperm. As in the case of many endangered species, information such as distribution and listing status can be obtained from state or federal agencies.

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Cryopreservation of Sperm of Striped Bass and White Bass

George G. Brown and Lynda D. Brown

Introduction

In hybrid bass aquaculture of striped bass *Morone saxatilis* and white bass *Morone crysops* considerable effort has produced techniques allowing yr-round culture (Bayless 1972, Woods and Sullivan 1992, Woods et al. 1993, Kohler et al. 1994, Sheehan et al. 1996). Procedures necessary to supplement these techniques are short-term (refrigerated) and long-term storage (cryopreservation) of striped bass and white bass sperm. Although short-term storage has been adequately performed, cryopreservation is more suitable when storage of semen would exceed 2 wk.

The practice of cryopreservation includes cooling, freezing and thawing of semen in a manner which maintains viable spermatozoa capable of fertilizing eggs. Performing this task involves: 1) an appropriate cryoprotectant solution to penetrate and dehydrate the cells to minimize cellular disruption and membrane damage from ice crystal formation; 2) the lowering of temperature at a proper cooling rate to -196 ^{o}C, the temperature of liquid nitrogen (LN_2), and 3) proper procedures to prevent damage during thawing. During one or more steps in this process, the percentage of viable spermatozoa can be reduced, so most techniques presently being developed attempt to moderate all problems to maintain a high percentage of viable spermatozoa. Therefore all steps in the freeze-thaw process are important and should be carefully performed.

Many successful cryopreservation procedures are complicated and use sophisticated cryoprotectants and state-of-the-art equipment, such as expensive controlled-rate freezers. On the other hand, simple procedures are also successful. With aquaculture species such as striped bass or white bass, procedures are needed that can be used in hatchery or field conditions with minimal expenditure. The methods and suggestions described here have proven successful and should aid and benefit culture of hybrid bass. These methods should also be considered as initial procedures that can be improved with additional studies.

Background for Cryopreservation of *Morone* Species

A number of cryoprotectants have been used to cryopreserve fish semen. Blaxter (1953) froze slices of herring testes in glycerol and carried out successful fertility studies. Other cryoprotectants include dimethyl sulfoxide (Erdahl et al. 1984), mannitol and dimethyl sulfoxide (Mounib et al. 1968) and sorbitol (Brown and Mims unpublished data). Cryoprotectants are used to reduce formation of ice crystals that can cause cell damage. This is obtained by using cryoprotectant solutions to penetrate the cells to allow dehydration during the freezing process. These mechanics and cryoprotectants are described in excellent reviews (Scott and Baynes 1980, Harvey 1982, Stoss and Donaldson 1982). Although several cryoprotectant procedures are available, the one that proves workable for the species of interest should be used until better cryoprotectants are found.

The semen of striped bass was cryopreserved with dimethyl sulfoxide (DMSO) and successfully used in fertility studies by Kerby (1982) and Brown et al. (unpublished data). Kerby

Brown, G. G. and L. D. Brown. 2011. Cryopreservation of Sperm of Striped Bass and White Bass. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 430-438.

also used the cryoprotectants glycerol, ethylene glycol and propylene glycol, but these were not successful in fertilization studies, and poor sperm motility was observed after thawing. Brown et al. (unpublished data), used 4DMSO for cryopreservation of striped bass semen and obtained successful fertilization and development in a hybrid cross with white bass eggs. These initial studies resulted in 6% fertility, compared to 2% fertility with control semen. In a study using 4% DMSO, Sheehan et al. (1996) successfully cryopreserved striped bass and white bass semen and obtained fertilization and development with white bass eggs. The results with striped bass semen were variable with average development ranging from 45 to 100% of control values. Average development in tests using white bass eggs fertilized with cryopreserved white bass semen ranged from 22 to 48% of control values.

Materials and Procedures for Storage and Cryopreservation

Semen of striped bass and white bass can be collected and cryopreserved in the laboratory with minimal equipment. Careful procedures are needed and cooling equipment and samples are important. Supplies and suggestions for semen cryopreservation for these two species are discussed below.

Controlled-rate Freezers

Such equipment is expensive, but cooling rates as rapid as -20 °C per min can be routinely controlled through the critical latent heat of fusion. Experiments in our laboratory have shown that the cooling rate of 0.5-mL and 5-mL straws packed in dry ice is within the range of acceptable rates for successful cryopreservation

Collection Containers

Semen can be collected in syringes, plastic bags, or tissue culture flasks. Syringes are useful if semen is to be cryopreserved soon after collection. Otherwise, plastic bags or flasks are preferable because an air space is present. Plastic bags (e.g. Zip Lock®) are cheap, clean, and hold large volumes of semen, but can allow stratification of semen which leads to oxygen-deprived zones. If several hr will elapse before cryopreservation procedures are initiated, tissue culture flasks are more suitable due to level storage on crushed ice or refrigerator shelves. Tissue culture flasks are more expensive than plastic bags but can be reused if rinsed (they should not be washed with detergent). Whatever type of collection container is used, the semen of striped bass and white bass must not be exposed continuously with external air (in other words, the lids of the flasks must be tightened). External air (presumably too much oxygen) will cause deterioration of samples leading to energy loss, membrane damage and reduction of sperm motility.

Extenders

We have evaluated three extenders for use with *Morone* semen (Table 1). As with semen samples, extenders must be stored on “wet” ice (ice allowed to warm to 0 °C after removal from freezer). Although all three extenders have been used for cryopreservation studies, use of extender #4H or extender #7 is recommended. These extenders can be prepared and stored frozen for yr.

Table 1. Chemical composition of extenders used to dilute semen of *Morone* species.

Ingredient	Extender #4H	Extender #7	Extender #13
$CaCl_2 \cdot 2H_2O$	0.103 g	0.103 g	- -
$MgCl_2 \cdot 6H_2O$	0.220 g	0.220 g	- -
$NaHCO_3$	0.235 g	0.235 g	- -
KCl	2.558 g	2.558 g	- -
NaCl	5.780 g	5.780 g	8.600 g
Glucose	10.0 g	- -	- -
Pyruvate	- -	6.000 g	- -
Citric acid	0.100 g	0.100 g	- -
HEPES buffer[a]	2.380 g	2.380 g	- -
Double-distilled water	1000 mL	1000 mL	1000 mL
KOH solution[b]	10 mL	10 mL	- -
Antibiotics[c]	10 mL	10 mL	10 mL
pH	7.6	7.6	7.6
mOsmol/kg	~ 310	~ 310	~ 310

[a]HEPES buffer, 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid.
[b]Potasium hydroxide solution, 1.27 g/100 mL.
[c]Penicillin-streptomycin (Sigma Chemical Corp. St. Louis, Missouri; P0906) 5000 units of penicillin, 5 mg streptomycin per mL of 0.9% NaCl in sterile water.

Preparation and Ice Storage

All samples, cryoprotectant ingredients, extenders, and supplies should be cooled before use. An ice chest (8 to 10 L) is useful for the storage of supplies and extenders. Additional ice chests are useful for storage of extended semen in cryoprotectant medium, and for holding of crushed dry ice. A convenient system is the storage of supplies in plastic or metal pans sitting on the ice.

Freezing Straws

Cryoprotectant straws (0.5 mL) can be obtained from several suppliers. These should have the standard “wick and powder” seal at one end. The straw is filled by using an automatic or manual pipette attached to the powdered end. The prepared sample is siphoned until it contacts the powder, whereupon the other end is sealed after ensuring the creation of a sufficient air space in the straw. Sealing can be accomplished with Critoseal® (a “putty-type” sealant for hematocrit tubes, TS Scientific, Perkasie, Pennsylvania) or by use of heat. If large 5-mL drinking straws (clear, nonflexible plastic straws available at commercial outlets and fast food establishments) are to be used, an adequate method for filling is to use a 5-mL syringe with 10 cm of polyethylene tubing attached to an 18-gauge needle. An air space should be left at each end of the large straws to prevent rupture during freezing.

Dry Ice

Dry ice (solid carbon dioxide) is available commercially or can be produced from carbon dioxide cylinders that discharge through a bottom pipe. Dry ice blocks can be crushed with a

wooden mallet and a burlap bag, or in a commercial ice crusher. With use of a carbon dioxide cylinder, a dry ice maker can be attached. This system produces a block that can be easily crushed in a gloved hand.

Liquid Nitrogen Storage Dewars

Two LN_2 dewars are necessary. A 2-L scientific Thermo-Flask (Lab-Line Instruments Inc., Melrose Park, Illinois) to drop straws into during the preparation is useful, but a less expensive container is a commercial 4-L plastic thermos or a 2-L tin can packed in styrofoam. A larger 20-L cryobiological storage chamber dewar for LN_2 is necessary for organizing and storing of samples over long periods of time. Such a storage chamber with six canisters can hold 1,000 or more 0.5-mL straws or 300 5-mL straws. For transport, straws can be packed in crushed dry ice and stored. Canes can be purchased to hold straws in canisters, but they take up space. If bulk freezing is performed, the canisters can be filled with straws. Protective gloves are necessary when handling samples.

Collection and Treatment of *Morone* Semen Before Cryopreservation

The semen must be carefully collected to avoid contamination with feces, urine or water. Dilution with water will activate the spermatozoa and reduce the viability. Tissue culture flasks are convenient for collecting milt as they can be placed under the genital opening during the expelling of semen. The flasks must be cooled on wet ice (ice bath). If the semen is not cooled, warm temperatures (5 °C and above) can activate or deteriorate the spermatozoa. Semen cooled on freezer ice, which is below 0 °C, may also suffer damage. Spermatozoa store energy in the form of high energy phosphagens and adenosine triphosphate. Warmer temperatures speed up the metabolism of these compounds leading to an irreversible degradation of the semen (Robitaille et al. 1986, Morisawa and Ishiba 1987, Robitaille et al. 1987a, 1987b). Cooling on ice can slow this process.

Evaluation of Milt and the Use of Extenders Before Cryopreservation

Semen of *Morone* species should be analyzed before cryopreservation. A useful and convenient criterion is sperm motility (Billard and Cosson 1992). Striped bass and white bass each have a high sperm concentration (50 to 125 x 10^9 spermatozoa per mL). A small drop placed on a coverslip and activated with a drop of pond water will show a sperm dispersal within the drop that can be observed with a dissecting microscope or the trained naked eye (Brown and Mims 1995). Glass microslides should not be used unless cleaned. The testing of sperm motilty directly on an uncovered coverslip is recommended. Commercial glass microscope slides frequently have oil deposits that can inhibit or in some cases prematurely activate sperm motility. Glass microslides covered with a coverslip can be misleading because the pressure of the coverslip can inhibit sperm motility (Clapper and Brown 1981). If coverslips are used on microscope slides, they must be suspended by clay “feet” or a layer of vaseline.

Use of Extenders

The philosophy for the use of extenders is practical. Whether or not to extend the milt of *Morone* species is dependent on the time of storage before cryopreservation. If chilled milt is not extended within a few hr of collection it will begin to deteriorate (Brown et al. unpublished data). If semen is to be used 3 to 4 hr after collection, it should be diluted with a suitable extender.

Recommended dilution with extenders is 1:2 (semen:extender) for striped bass and 1:1 for white bass (Table 1). The extender should be added directly to the milt with gentle swirling until completely mixed. When storing samples in flasks, the lids should remain tightened with no more than 5 to 6 mL of semen or extended mixture in each flask.

Cryopreservation Procedures

Dimethyl sulfoxide is a suitable cryoprotectant for *Morone* sperm. To date, our best results have been obtained with 4% DMSO mixed with trehalose and extender (Table 2). Extended semen and cryoprotectant medium were stored on ice. One milliliter of the cryoprotectant was placed in several 5-mL plastic centrifuge tubes. An automatic pipette with cooled tips was used to place 0.25-mL aliquots of extended semen into each centrifuge tube. Alternatively, larger amounts of semen and cryoprotectants can be mixed (for example, 1 mL of semen with 4 mL of cryoprotectant media). The mixture of semen and cryoprotectant was allowed to equilibrate for 10 to 15 min, and 0.5 mL of the mixture was siphoned into a freezing straw that was sealed and placed on crushed dry ice. After 15 min, each straw was placed in a thermos containing LN_2. The straws were stored in the vapor portion of a cryobiological storage container until use in fertility studies.

Table 2. Composition of media used to cryopreserve semen of *Morone* species.

Ingredient	Standard (4% DMSO)	Standard (4% DMSO)	Concentrated (16% DMSO)
DMSO	40 µL	0.4 mL	1.6 mL
Trehalose[a]	100 µL	1.0 mL	4.0 mL
Extender[b]	860 µL	8.6 mL	34.4 mL
Total volume	1000 µL	10.0 mL	40.0 mL

[a]100 mg/mL of extender.
[b]Extender composition (see Table 1).

Thawing and Fertility Studies of Cryopreserved Semen

Straws containing cryopreserved samples were thawed under running tap water (20 to 22 °C) for 5 to 10 sec. The ends were snipped off and the contents were drained into 5-mL centrifuge tubes. Samples were examined for sperm motility or were mixed with freshly stripped eggs. The best sperm motility resulted when the contents of the straws were slushy or not completely thawed. This procedure was the same whether fertilizing white bass eggs with thawed semen of striped bass or white bass.

Suggested Improvements of the Procedures

A suggested improvement in the cryopreservation of striped bass and white bass semen is to avoid dilution with extenders before mixing with the cryoprotectant medium. Semen in previous studies have routinely been diluted with extenders either 1:2 (semen:extender) for striped bass semen or 1:1 for white bass semen. This step could be avoided when semen is

collected immediately before cryopreservation. The reason for dilution is to prevent degradation of sample due to storage before the cryopreservation procedures. If cryopreservation is to be performed immediately, dilution may not be necessary. The result of no dilution would be to increase the concentrations of spermatozoa and seminal fluid.

A sec suggested improvement is to use a higher initial percent of DMSO (16% instead of 4%). This would allow more semen to be preserved in a smaller amount of the cryoprotectant medium. This has been successfully carried out with paddlefish *Polyodon spathula* where semen is dilute (Mims et al., this volume). With paddlefish, 1.0 mL of 16% DMSO (Table 2) was mixed with 3.0 mL of semen resulting in a final 4% DMSO mixture, which contained a higher concentration of spermatozoa and seminal fluids. When using this procedure, the toxic effect of DMSO during mixing with semen must be determined.

These suggestions would provide a higher concentration of spermatozoa per straw. A higher concentration would allow a higher number of viable spermatozoa, but may also increase the percentage of spermatozoa surviving cryopreservation. Because the seminal fluid concentration is also increased, spermatozoa would be bathed in a more natural medium during the freezing and thawing process, possibly aiding in the prevention of premature activation and sperm membrane damage. This procedure could thus increase the number of viable sperm in a 0.5-mL straw by as much as ten-fold when compared to the routine procedure. Larger straws (5-mL) would supply larger quantities of spermatozoa that would be useful in mass production. Larger straws were used in our paddlefish studies and produced not only higher amounts of spermatozoa, but also a higher percent of viable spermatozoa.

Other suggested research areas include the study of polyols (Utsumi et al. 1992), which are a cheap and easy to use cryoprotectant for *Morone* sperm. Polyols have been shown to penetrate cells faster than DMSO and to be less toxic, but may be difficult to remove from the cells. Brown and Mims (unpublished data) cryopreserved paddlefish semen in sorbitol, but were not able to obtain fertilization.

Summary

Methods for cryopreservation of *Morone* semen have been developed and demonstrated. Successful fertilization using these methods was performed. However, this represents initial approaches and improvement of procedures for use in the aquaculture industry is needed. Development of reliable techniques to store, cryopreserve, and transport gametes will contribute immensely to the culture of hybrid striped bass.

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Annotated Bibliography of Developments in the Last Decade

Since the publication of this book in 2000 *Morone* cryopreservation has remained an active field of research. Publications have been prolific with this area of sperm cryopreservation moving towards commercialization. This is due to the sole reliance of the hybrid striped bass industry on wild broodstocks. Cryopreservation could help to reduce the need to collect wild broodstock. There is a new chapter in the second edition of this book that discusses sperm cryopreservation in striped bass in further detail.

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New Chapter

Sperm Cryopreservation in Biomedical Research Fish Models

Huiping Yang and Terrence R. Tiersch

Introduction

With the continued development of genomic tools conserved and species-specific molecular mechanisms can be identified, and comparative studies among vertebrate species are becoming commonplace for human biomedical research. As the largest class of vertebrates, bony fishes offer almost unlimited versatility for research. Generally, model fish require features such as small body size, high fecundity, ease of culture, and most importantly, specific characteristics for particular research topics. Currently, the most widely used fish models are zebrafish *Danio rerio* (Driever et al. 1994, Kari et al. 2007), medaka *Oryzias latipes* (Wittbrodt et al. 2002), live-bearing fishes of the genus *Xiphophorus* (Walter and Kazianis 2001), mummichog *Fundulus heteroclitus* (Atz 1986, Burnett et al. 2007) and pufferfish *Fugu rubripes* (Elgar et al. 1996). With extensive studies using these fish models, thousands of specific strains and lines have been created, and are currently housed worldwide as live animals in resource centers, such as the Zebrafish International Resource Center (University of Oregon, Eugene) which holds around 1080 inbred, transgenic, knockout and mutant strains; the University of Georgia (Athens), which holds several inbred and transgenic medaka lines, and the *Xiphophorus* Genetic Stock Center (Texas State University, San Marcos), which holds 61 inbred lines (of which most have been maintained for 50 to more than 100 generations) of 24 species. Preservation of the genetic resources of these and other valuable fishes presents significant and urgent challenges. Gamete or embryo cryopreservation is a useful approach to address these challenges.

Ideally, a conservation program should include the preservation of sperm, eggs, embryos and larvae to secure the revival of species or strains. Currently, cryopreservation techniques in fish are mostly applied to sperm. Cryopreservation has not been successful for eggs and early embryos because of their large size, high lipid content, polar organization (Blesbios and Labbe 2003), and membrane impermeability (Hagedorn et al. 1998). Sperm cryopreservation in fish mostly has focused on large-bodied aquaculture species, such as salmonids, carps, and catfishes, and only several studies have addressed aquarium fishes. Due to the small body sizes and limited sperm availability, sperm cryopreservation in aquarium fishes presents challenges, such as in experimental design, gamete collection, and artificial fertilization, especially in live-bearing fishes (Tiersch 2001).

We intend for this review to provide an overview of sperm cryopreservation in zebrafish, medaka, and *Xiphophorus*, the most important biomedical fish models, and hope it can serve as a template for research on other aquarium fishes. These three groups possess some distinct differences. For example, they occur naturally in two habitats: strict freshwater (zebrafish and *Xiphophorus*) (Hawkins et al. 2001) and brackish-water-accommodated freshwater (medaka) (Inoue and Takei 2002), and they have two reproduction modes: external fertilization (zebrafish and medaka) and internal fertilization (*Xiphophorus*).

Yang, H. and T. R. Tiersch. 2011. Sperm Cryopreservation in Biomedical Research Fish Models. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 439-454.

Characteristics of Zebrafish, Medaka, and *Xiphophorus* Fishes

Habitat

The natural environment can influence reproductive modes and traits. Zebrafish, a strict freshwater species, naturally occurs in slow or still freshwater systems (rivers, small streams, pools, and rice paddies) in a range extending from Pakistan in the west to Myanmar (Burma) in the east, and from Nepal in the north to the Indian state of Karnataka in the south (Engeszer et al. 2007). Medaka is a euryhaline species, distributed widely in freshwater habitats of China, Japan, and Korea (Naruse 1996, Naruse et al. 1993), and are also found in brackish water (Miyamoto et al. 1986). Unlike zebrafish and *Xiphophorus* fishes, medaka can be acclimated from freshwater to brackish and even sea water, and can reproduce in fresh water and brackish water (Inoue and Takei 2002). Fishes of the genus *Xiphophorus* naturally live in backwaters in Mexico, Guatemala, Belize and Honduras along the Gulf coast of Mexico (Kallman 2001), and are also strict freshwater species.

Reproduction

Zebrafish, medaka, and *Xiphophorus* fishes are all dioecious. Zebrafish reproduce by external fertilization. Eggs and sperm are released into environmental water to fertilize and develop, and fry can hatch at about 24 hr after fertilization at 26-28 ºC (Laale 1977).

In medaka, the male and female participate in mating behavior before spawning, eggs are expelled as a cluster attached to the belly of the female, and become attached to floating aquatic plants. Fry hatch after 7-10 d at 26 ºC (Yamamoto 1975a).

For *Xiphophorus* fishes, the reproduction mode is internal fertilization. Males are distinguished from females by the secondary sexual organ, a modified anal fin called the gonopodium, and other phenotypes such as body color and in some species, a sword-like tail. After copulating with the male, female *Xiphophorus* can store sperm for months and subsequently produce broods at approximately 30-d intervals for 4-5 months without the presence of a male. The intervals between mating and the first brood are irregular and can vary between 26 and 90 d (Tavolga 1949).

Body Size, Availability of Testis, and Sperm Production

Zebrafish, medaka, and *Xiphophorus* are all small-bodied fishes with lengths of less than 5 cm, and therefore the availability of sperm by stripping is limited to several microliters. Accumulated data on body size, body weight, testis weight, and the sperm production per mg of testis weight (by dissection) for these species are listed in Table 1. Basically, for all of these species correlation analysis has showed that body weight, body length, and testis weight were positively correlated with each other.

Testis Morphology

Gonadal and gametic morphology of fishes have been studied for decades at anatomical or histological levels to identify annual reproduction cycles and length of breeding season, and have been used for analysis of evolution and phylogeny in bony fishes (Jamieson 2009, Parenti and Grier 2004). For zebrafish, medaka, and *Xiphophorus*, testis structure falls into three different types. Zebrafish possess what has been characterized as the anastomosing tubular type (Maack and Segner 2003), which is widely found throughout primitive teleost taxa (Parenti and Grier 2004). Medaka posses the type characterized as the restricted lobular testis (Grier 1976), in

which spermatogonia distribute into the distal ends of lobules. *Xiphophorus* fishes possess the type characterized as the restricted lobular testis (Grier et al. 1980), and the spermatozoa are formed into spermatozeugmata ("sperm bundles"), specialized structures with spermatid nuclei oriented outward toward the Sertoli cells.

Table 1. Basic biological characteristics and sperm production by crushing dissected testis in zebrafish *Danio rerio* (AB lines) from the Zebrafish International Resource Center, medaka *Oryzias latipes* from the University of Georgia (a strain originally derived native to Southern Japan, obtained from Carolina Biological), and four *Xiphophorus* species from the *Xiphophorus* Genetic Stock Center.

Species	Males used	Length (cm)	Weight (g)	Testis (mg)	Sperm /mg of testis (x10^6 cells)	Reference
Zebrafish	45	2.4 ± 0.2	0.295 ± 0.066	3.2 ± 2.0	7.7 ± 2.0	Yang et al. unpublished
Medaka	74	2.6 ± 0.2	0.311 ± 0.052	1.9 ± 0.6	2.0 ± 0.4	(Yang et al. 2010)
X. helleri	45	3.2 ± 0.3	0.630 ± 0.168	9.2 ± 5.5	5.4 ± 2.2	(Huang et al. 2004a)
X. couchianus	66	2.0 ± 0.2	0.184 ± 0.052	3.1 ± 1.0	5.4 ± 2.5	(Yang et al. 2009)
X. maculatus	117	2.5 ± 0.3	0.474 ± 0.149	7.1 ± 3.7	5.8 ± 2.8	Yang et al. unpublished
X. variatus	35	2.5 ± 0.2	0.298 ± 0.096	6.4 ± 3.4	2.4 ± 1.3	Yang et al. unpublished

Sperm Motility Activation

Fish spermatozoa are usually immotile while in the testis and seminal plasma in most species studied (Cosson 2004). Naturally, sperm are activated during spawning to fertilize eggs. For most fishes with external fertilization, osmolality is a dominant factor for activating sperm (Morisawa and Suzuki 1980). In general, for freshwater fish species, motility is initiated by exposure to hypotonic solutions, and for marine species, motility is initiated by exposure to hypertonic solutions (Morisawa and Suzuki 1980). These activation modes match the environment where sperm function during spawning. For example, zebrafish inhabit fresh water, and sperm motility can be activated by exposure to hypotonic solutions (Yang et al. 2007a).

Although they inhabit freshwater, as a euryhaline fish medaka show a mode for activation of sperm motility different from typical freshwater fishes. Sperm motility in medaka could be initiated by distilled water without electrolytes (25 mOsmol/kg) and by Hanks' balanced salt solution with osmolalities spanning from 92 to 686 mOsmol/kg, a range including hypotonic, isotonic, and hypertonic osmolalities (Yang and Tiersch 2009). Upon activation, the sperm could remain continuously motile for as long as one week. These sperm characteristics are distinct from those in most freshwater and marine fishes whether they are external or internal fertilizers, and are potentially representative of other euryhaline fishes.

As viviparous fishes, *Xiphophorus* possess a completely different mechanism for activation of sperm motility. Within the testis, sperm are compacted into spermatozeugmata and are immotile. When suspended in electrolyte solutions isotonic to seminal plasma, the sperm become motile, but in either hypertonic or hypotonic solutions, they are quiescent (Morisawa and Suzuki 1980, Yang et al. 2006). This mode of motility activation has been described in *Xiphophorus* species (Huang et al. 2004a, Yang et al. 2006) and Western mosquitofish *Gambusia affinis* (Morisawa and Suzuki 1980), but may not be representative of all viviparous fishes, such as the redtail splitfin *Xenotoca eiseni* of the family Goodeidae (our unpublished observations). This is because viviparity has emerged independently throughout vertebrate evolution in a

variety of groups (Long et al. 2008), thus presenting a diverse array of adaptations to address the problem of internal fertilization (DeMarais and Oldis 2005, Ryan 1998).

Development of Protocols for Sperm Cryopreservation

Cryopreservation is a technique involving a series of steps including sample collection, sperm dilution in extender, cryoprotectant selection, freezing, storage, thawing, and viability detection (Tiersch 2000). Development of protocols for requires suitable choices at each step and consideration of the interactions among the factors. The success of cryopreservation can be demonstrated by fertilization and production of live offspring. Due to the multiple steps and their interactions, errors at each step can accumulate and lead to considerable losses of viable cells. Thus, careful attention should be given to the details at each step, and care should be taken to reduce or eliminate sources of uncontrolled variation (Leibo 2000). Protocols of sperm cryopreservation can vary because of species-specific differences in sperm size, shape, and biochemical characteristics. Development of protocols for sperm cryopreservation for zebrafish, medaka, and *Xiphophorus* fishes are summarized in Table 2 (next page). We review and compare below the current status of sperm cryopreservation in these fishes.

Sperm Collection

Sperm of aquarium fishes can be collected by stripping or by crushing of dissected testis. Due to their small body size, the availability of sperm by stripping is limited to 1-2 µl. For zebrafish and medaka, the stripped samples are composed of highly concentrated single cells. For *Xiphophorus* fishes, stripped sperm samples are a mixture of single sperm cells, and broken and intact bundles (our unpublished observations). Collection by stripping of sperm samples avoids the killing of valuable fish, and individual males can be sampled repeatedly. However, to maximize the volume of sperm available, especially to allow experimental replication, crushing of dissected testis has been used for sperm collection in most published studies.

Extender Selection

Dilution of sperm in extender solution is necessary for cryopreservation. The role of the extender is to retain the functional capability and fertilizing ability of sperm by controlling the pH, osmolality, ion concentration, and in some cases, the supply of energy. An understanding of sperm activation and motility is necessary to formulate extender solutions. Usually, extenders are balanced-salt buffers with specific pH and osmolality to prevent sperm motility activation. Although, many extenders have been reported for sperm cryopreservation, there are not always observed differences in post-thaw motility (Stoss and Holtz 1981).

For zebrafish, osmolality is the dominant factor to control motility activation as stated above. Once activated by hypotonic osmolality, sperm have a short burst of peak motility (30 sec to 2 min) (Yang et al. 2007a). Thus, sperm need to be held in extender that is isotonic to the plasma osmolality (~300 mOsmol/kg) to inhibit activation. In zebrafish, three different extenders (Ginsburg, BSMIS, HBSS) have been reported for use with cryopreservation (Appendix) and all of them functioned well to retain post-thaw fertility (Draper et al. 2004, Harvey et al. 1982, Morris et al. 2003, Yang et al. 2007a). With respect to composition, Ginsburg extender contains a specific brand of powdered skim milk (Draper et al. 2004, Harvey et al. 1982) which is not readily available worldwide, and impedes the observation of sperm motility and morphology,

Table 2. Summary of previous studies in sperm cryopreservation of zebrafish, medaka, and *Xiphorphorus* fishes.

Extender	Cryoprotectant	Packaging	Cooling method	Thawing method	Assessment	Reference
Zebrafish						
Ginsburg	8.3% Methanol	Capillaries	16 ºC/min from 4 to -35 ºC	RT[a]	51 ± 36% fertilization[b]	(Harvey et al. 1982)
BSMIS	10% Dimethyl acetamide	Capillaries	Placement on dry ice for 30 min	Dilution with 20-x volume of RT extender	9-14% fertilization	(Morris et al. 2003)
Ginsburg	8.3% Methanol	Cryovials	Placement on dry ice for 20 min	33 ºC for 8 sec	28 ± 18% fertilization	(Draper et al. 2004)
HBSS	8% Methanol	French straws	10 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	33 ± 20% fertilization	(Yang et al. 2007a)
Medaka						
Fetal bovine serum	10% Dimethyl formamide	Cryovials	Placement in vapor of liquid nitrogen for 10 or 20 min	30 ºC for 0.5-1 min, dilution in 2-x BSS solution	96-100% fertilization	(Aoki et al. 1997)
0.6-M sucrose	10% Dimethyl sulfoxide	Capillaries	Placement on dry ice for 20 min	Holding between fingers	85% fertilization	(Krone and Wittbrodt 1997)
HBSS 350	10% Methanol	French straws	10 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	70 ± 30% hatching	(Yang et al. 2010)
Xiphophorus helleri						
HBSS 240-300	10% Dimethyl sulfoxide	French straws	45 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	29 ± 8% (post-thaw motility)	(Huang et al. 2004a)
HBSS300	14% glycerol	French straws	20-35 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	77 ± 3% (post-thaw motility)	(Huang et al. 2004b)
HBSS310 HBSS500	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	1-3 of 15 females produced offspring	(Yang et al. 2007b)
Xiphophorus couchianus						
HBSS 240-300	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 7 sec	78 ± 3% (post-thaw motility)	(Huang et al. 2004c)
HBSS500	14% glycerol	French straws	25 ºC/min from 5 to -80 ºC	40 ºC for 5 sec	2 of 15 females produced offspring	(Yang et al. 2009)

RT: Room temperature.

This number is not an actual value, it is relative to the control hatch which was 71 ± 5%; thus the actual rate was: 0.71*51 = 36%.

especially after thawing. The other extenders, HBSS and BSMIS, which do not contain milk, are more suitable choices for sperm cryopreservation of zebrafish.

Medaka sperm have a long swimming duration upon activation (Yang and Tiersch 2009). Therefore, the extender is not necessarily required to inhibit sperm motility. Sperm suspended in HBSS at osmolalities ranging from 92 to 373 mOsmol/kg remained continuously motile for 7 d (Yang and Tiersch 2009). Therefore, HBSS can be a candidate extender for medaka sperm across this range of osmolalities. In artificial insemination of medaka eggs, a balanced salt solution (BSS, see Appendix) with an osmolality of 230 mOsmol/kg has been used to retain fertility of the gametes (Iwamatsu 1983, Kinoshita et al. 2009). For sperm cryopreservation, different extenders have been used and have yielded comparable post-thaw motility and fertility, including BSS (Kinoshita et al. 2009), fetal bovine serum (Aoki et al. 1997), 0.6 M sucrose (Krone and Wittbrodt 1997), and HBSS with an osmolality of 350 mOsmol/kg (Yang et al. 2010). Systematic investigation is needed to test whether different osmolalities and composition of extenders can produce differences in sperm viability during sperm cryopreservation.

For *Xiphophorus* fishes, motility can be activated by isotonic solutions (~310 mOsmol/kg), rather than by hypotonic or hypertonic, and upon activation sperm can remain continuously motile for as long as one week (Huang et al. 2004a, Yang et al. 2006). Based on these characteristics, HBSS at 310 mOsmol/kg was used as the extender for sperm cryopreservation, and high motility was obtained after thawing for *X. helleri* (58 ± 7%) (Huang et al. 2004b) and *X. couchianus* (78 ± 3%) (Huang et al. 2004c). Alternatively, HBSS at an osmolality of 500 mOsmol/kg was used as extender for *X. helleri* because sperm immobilized at this osmolality could be re-activated by changing into isotonic osmolality (Yang et al. 2006). Sperm of *X. helleri* extended in HBSS at an osmolality of 500 mOsmol/kg were also cryopreserved, and high motility (~55%) was obtained after thawing (Yang et al. 2006). It was hypothesized that osmotic immobilization of sperm prior to freezing by use of hypertonic HBSS at 500 mOsmol/kg could conserve sperm energy capacity, and consequently could provide an advantage for internal fertilization compared to sperm cryopreserved using isotonic HBSS, especially if the sperm were to be stored prior to cryopreservation or insemination. Through artificial insemination, sperm cryopreserved at 310 and 500 mOsmol/kg each HBSS produced verified (hybrid) offspring (Yang et al. 2007b).

Because of the limited availability of sperm from aquarium fishes, dilution of samples with extender is useful to maximize the volume available for subsequent analysis. Therefore, dilution ratios of testis weight (mg) : HBSS volume (µl) of 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 were evaluated with sperm of *X. helleri*. Motility of samples diluted at 1:50, 1:100 and 1:200 was found to be not significantly different before freezing or after thawing, but declined significantly at ratios of higher than 1:200 (Huang et al. 2004b). For zebrafish and medaka, no similar evaluation of dilution ratio has been reported, but ratios of testis weight (mg) : HBSS (µl) of 1: 50-80 were used for sperm cryopreservation, and high percent motility and fertility were obtained in thawed sperm (Yang et al. 2007b). Extreme dilution of samples has been found to reduce sperm motility in animals such as mammals (Harrison et al. 1978), rainbow trout *Oncorhynchus mykiss* (Billard 1983, Scott and Baynes 1980) and eastern oysters *Crassostrea virginica* (Paniagua-Chavez et al. 1998), and remains a topic for study in aquarium fishes.

Cryoprotectant Selection

In sperm cryopreservation, cryoprotectants are additives necessary for protection against freezing damage due to intracellular ice crystal formation and excessive dehydration. Usually

cryoprotectants are grouped into two categories: permeating cryoprotectants (e.g., dimethyl sulfoxide (DMSO), methanol, and glycerol) and non-permeating cryoprotectants (e.g., egg yolk, milk, and proteins). A variety of cryoprotectants have been evaluated for different species (Fuller 2004), and selections are usually determined experimentally. Theoretically, the higher the concentrations of cryoprotectant, the better the protection to sperm cells should be during cryopreservation. However, high concentrations of cryoprotectants can be toxic or lethal to sperm cells. The optimum concentration should be a value which balances these two effects. Usually, concentrations of 5 to 20% were chosen for experiments with sperm cryopreservation in aquarium fish.

For zebrafish, the toxicity of DMSO, N,N-dimethyl acetamide (DMA), methanol, and glycerol at concentrations of 5, 10, and 15% were evaluated with sperm cells. Glycerol was the most toxic, and was eliminated from further consideration. The other three chemicals were used for sperm cryopreservation, and analysis of post-thaw motility showed that methanol at a concentration of 8% was the best choice (Yang et al. 2007a). This was also the choice in two earlier studies (Draper et al. 2004, Harvey et al. 1982). In addition, DMA (10%) was used as a cryoprotectant for zebrafish sperm (Morris et al. 2003), but the fertilization level after thawing (9-14%) was lower than that observed (28-51%) when methanol was used as cryoprotectant (Draper et al. 2004, Harvey et al. 1982, Yang et al. 2007a).

For medaka, 10% DMSO and 10% dimethyl formamide (DMF) were used to cryopreserve sperm, and after thawing yielded good motility (78-100%) and hatching (82-100%) (Aoki et al. 1997, Krone and Wittbrodt 1997). A recent study systematically evaluated cryoprotectant and cooling rate for medaka sperm cryopreservation (Yang et al. 2010) and compared them with the two previous publications. The evaluation of acute toxicity of six cryoprotectants, methanol, 2-methoxyethanol (ME), DMSO, DMA, DMF, and glycerol showed that methanol and ME (5% and 10%) did not change the sperm motility after 30 min; DMSO, DMA, and DMF (10% and 15%) and glycerol (5%, 10% and 15%) significantly decreased the motility of sperm within 1 min after mixing. Based on these results, methanol and ME were selected as cryoprotectants (10%) to evaluate with different cooling rates (from 5 to 25 °C/min) and were compared in parallel to DMSO and DMF (10%) because of their use as cryoprotectants in previous publications (Aoki et al. 1997, Krone and Wittbrodt 1997). The highest post-thaw motility (50 ± 10%) was observed at a cooling rate of 10 °C/min with methanol as cryoprotectant. Comparable post-thaw motility (37 ± 12%) was obtained at a cooling rate of 15°C/min with ME as cryoprotectant. With DMF, post-thaw motility at all cooling rates was ≤ 10% which was significantly lower than that of methanol and ME. With DMSO, post-thaw motilities were ≤ 1% at all cooling rates, and significantly lower compared to the other three cryoprotectants. Fertility testing of thawed sperm cryopreserved with 10% methanol at a rate of 10 °C/min showed average hatching of 70 ± 30% which was comparable to that of fresh sperm (86 ± 15%).

For *Xiphophorus*, DMSO, DMF, DMA, glycerol, propylene glycol, methanol, and sucrose were evaluated as cryoprotectants, each with final concentrations of 6% and 10% (v/v). The results indicated that DMSO and glycerol were suitable cryoprotectants, and further evaluation of these two cryoprotectants at different concentrations showed that glycerol was better than DMSO in retaining motility and prolonging storage time for *X. helleri* sperm after thawing, and the effective concentration for glycerol was 14% (Huang et al. 2004b). Also, glycerol showed the best results for cryopreservation of *X. couchianus* sperm with a

concentration of 14% (Huang et al. 2004c), and for osmotically immobilized sperm from *X. helleri* (Yang et al. 2006, Yang et al. 2007b).

After mixing with sperm, cryoprotectants require time for equilibration to penetrate the cells. This is a dynamic process depending on the permeability of sperm cells, cryoprotectants, and their concentrations. For zebrafish, based on toxicity analysis, a 10- to 20-min equilibration time was chosen (Yang et al. 2007a). For medaka, based on the toxicity analysis an equilibration time of less than 30 min was used (Yang et al. 2010). For *Xiphophorus*, equilibration times of 10, 20, 30, 60, and 120 min were evaluated for sperm of *X. helleri* and *X. couchianus*. An equilibration time of less than 30 min yielded the highest post-thaw motility in each species, but there was no consistent difference across equilibration times ranging from 10 to 120 min (Huang et al. 2004b, Huang et al. 2004c).

Packaging of Samples for Freezing

In sperm cryopreservation, packaging of samples for freezing and storage is important to standardize the cooling rate, and to assure sample identification. Currently, several different kinds of containers have been used for aquarium fishes such as plastic cryovials, glass tubes and ampules, and plastic straws. The different materials and shapes of these containers result in different heat transfer properties during freezing and thawing. Even for the same style of container, differences can exist with products from different manufacturers, which can result in variation of cooling or thawing rates. Therefore, it is necessary to standardize the packaging method to ensure that protocols will be repeatable especially in different laboratories.

For the aquarium fishes addressed in this review, the small volumes of sperm available limit the choices for sample packaging. In zebrafish and medaka, glass capillary tubes or cryovials were first employed in sperm cryopreservation (Aoki et al. 1997, Draper et al. 2004, Harvey et al. 1982, Krone and Wittbrodt 1997, Morris et al. 2003). Recently, to standardize protocols with potential for automation at high throughput, French straws were chosen for sperm packaging with the smallest commercially available volume (0.25 ml), and a more standardized protocol was developed with results comparable to previous studies (Yang et al. 2007a, Yang et al. 2010). For *Xiphophorus* fishes, French straws (also 0.25 ml) were used for sample packaging in all studies (Huang et al. 2004a, Huang et al. 2004b, Huang et al. 2004c, Yang et al. 2006, Yang et al. 2007b, Yang et al. 2009). Compared to capillary tubes or cryovials, the use of French straws has the following advantages: potential for use with automated filling and sealing equipment, sample identification by permanent printing of alpha-numeric labels or barcodes, sample biosecurity by complete sealing, and by virtue of thin high surface area-to-volume ratio, standardization of the cooling and thawing processes.

Cooling Rate Selection

Cooling rate is a crucial factor in sperm cryopreservation because it affects the osmotic and pH balance of intracellular and extracellular solutions during freezing. Theoretically, with an excessively slow cooling rate, osmotic equilibrium is maintained, and much of the freezable water leaves the cell resulting in excessive dehydration; with an excessively fast cooling rate, little or no freezable water leaves the cell, and thus large intracellular crystals can form, causing damage to the cell. Ideally, a balanced situation allows survival when the cooling rate is fast enough to minimize the time of exposure to concentrated solutions and yet is slow enough to minimize the amount of intracellular ice formation. Optimum cooling rates vary with different cryoprotectants and the physiology of sperm cells from different species, and can be determined

empirically by experimentation, or predicted by theoretical calculation using techniques such as differential scanning calorimetry (DSC). This technique can be used to estimate water permeability (Lp) at subzero temperatures and the activation energy of that process, and these values can be used to compute the amount of water loss in cells as a function of cooling rate and temperature, and predict the optimum cooling rate from such plots (Devireddy et al. 1998). The cooling process for aquarium fishes has been accomplished by use of the following methods: placement on dry ice, suspension in liquid nitrogen vapor, and controlled cooling with a programmable freezer. Dry ice and liquid nitrogen vapor are inexpensive and can be used in field situations, but the cooling rates are difficult to quantify and control. In contrast, programmable freezers offer high levels of control and reproducibility, but are expensive and difficult to use in the field.

For zebrafish, the cooling methods that have been reported are placement on dry ice (Draper et al. 2004, Harvey et al. 1982, Morris et al. 2003) and a programmable freezer (Yang et al. 2007a). With 8% methanol as the cryoprotectant and HBSS as extender in 0.25-ml straws, the suitable cooling rate was identified as 10 ºC/min (Yang et al. 2007a), and with Ginsburg buffer plus powdered milk as extender in 2-ml cryotubes 16 ºC/min was selected (Harvey et al. 1982).

For medaka, cooling was provided by use of liquid nitrogen vapor (Aoki et al. 1997) or dry ice (Krone and Wittbrodt 1997) without quantification. In our study, cooling rate, controlled by a programmable freezer, was found to be a sensitive factor for determining post-thaw motility of medaka sperm. A change of as small as 5 ºC/min in the cooling rate resulted in a significant change in post-thaw motility, and a cooling rate of 10 ºC/min was identified when 10% methanol was used as cryoprotectant in 0.25-ml straws (Yang et al. 2010).

For *Xiphophorus*, cooling rate was controlled by use of a programmable freezer. The results showed that 20 to 30 ºC per min was optimum when sperm were cryopreserved with 14% glycerol in 0.25-ml straws (Huang et al. 2004b, Huang et al. 2004c). The optimal value of cooling rate predicted by DSC agreed with the empirical results in *X. helleri* (Huang et al. 2004b, Thirumala et al. 2005), but not in *X. maculatus* for which the cooling rate was predicted as 47 ºC/min (Pinisetty et al. 2005).

Storage of Frozen Samples

Holding of frozen samples in liquid nitrogen (-196 ºC) in a storage dewar is a standard method for cryogenic storage of samples from aquarium fishes. During storage, the important considerations are sample identification, potential contamination, and inventory of frozen samples. The use of plastic or French straws for packaging, especially newer forms with high safety and durability, offer the advantages of permanent labeling by printer, and complete sealing which minimizes or prevents transfer of materials (e.g., sperm cells or bacteria) among samples stored in the same dewar (Morris 2005).

Thawing of Frozen Samples

Theoretically, the process of thawing is the reverse of freezing, and thus the damage that can occur during cooling can also occur during warming, primarily through formation of intracellular ice crystallization between -40 °C and 0 °C (Leung and Jamieson 1991). Thus, it is usually desirable to thaw cryopreserved samples rapidly to minimize the period of crystal propagation (termed “recrystallization”). Currently, systematic evaluation of thawing rates has not been reported for sperm cryopreservation of zebrafish, medaka, or *Xiphophorus*, probably because of the limited sample volumes. Generally, for frozen samples packaged in 0.25-ml

French straws, a 5-sec exposure within a 40 ºC water bath is practical and yields suitable motility and fertility after thawing in zebrafish and *Xiphophorus* fishes (Huang et al. 2004a, Huang et al. 2004b, Huang et al. 2004c, Yang et al. 2007a, Yang et al. 2006, Yang et al. 2007b, Yang et al. 2009, Yang et al. 2010). For frozen samples packaged in capillary tubes or cryovials in zebrafish and medaka, the thawing process has been performed by leaving samples at room temperature (Harvey et al. 1982), diluting with room temperature buffer (Aoki et al. 1997, Morris et al. 2003), holding within a 33 ºC water bath (Draper et al. 2004), or holding within the fingers (Krone and Wittbrodt 1997).

Viability Analysis of Cryopreserved Sperm

The purpose of cryopreservation is to obtain viable sperm which retain their fertility. Examination of the viability of cryopreserved sperm can include evaluation of morphology, membrane integrity, motility, ability to bind oocytes, and fertilization. Motility is the most widely used assay, but fertilization is considered to be the most informative.

Artificial insemination is necessary to test the fertility of cryopreserved sperm. This process includes a series of steps: egg collection, holding of eggs prior to fertilization, thawing of cryopreserved sperm, mixing of the sperm and eggs, activation of the gametes, fertilization confirmation, hatching of fertilized eggs, and offspring harvest and identification. For species with internal fertilization such as the *Xiphophorus* fishes, this process involves more complicated techniques such as the injection of sperm (2-4 µl) into the female reproductive tract and pregnancy confirmation. Factors related to females such as egg quality can also determine fertilization success. Therefore, development of standardized protocols for fertilization assays including collection and holding of eggs and sperm need be included in protocol development for sperm cryopreservation.

For zebrafish and medaka, artificial fertilization protocols have been established with fresh sperm, and can be directly modified to provide fertilization analysis of cryopreserved sperm (Westerfield 2005, Yamamoto 1975b). Eggs can be collected daily by squeezing of females or dissection (for medaka), held in isotonic buffer to retain fertility, and be mixed with a sperm suspension for fertilization. For zebrafish, after mixing of sperm and eggs, fresh water needs to be added to activate gametes for fertilization, but for medaka, this is not necessary because fertilization can occur in isotonic buffer as stated above. Fertilization and hatching are determined by assessing the percentage of developing embryos or hatched fry.

For *Xiphophorus* fishes, artificial insemination with cryopreserved sperm must consider points such as the use of virgin females (because female *Xiphophorus* can store sperm for successive broods), proper injection volume and technique, pregnancy monitoring for as long as 90 d, and confirmation of paternal contribution to offspring. Previous work has shown that centrifugation did not reduce post-thaw motility (Dong et al. 2006), and thus removal of the cryoprotectant from thawed sperm by washing is feasible although it may not be necessary (Yang et al. 2007b). Also, social interactions among females are another factor to be considered because they can influence maturation and brood timing (Earley 2006). Thus, based on these considerations, hybrid offspring produced through artificial insemination with cryopreserved sperm from *X. helleri*, were used for verification of paternity in the first report for viviparous fishes (Yang et al. 2007b). Following the same protocols, live young have been produced with cryopreserved sperm from *X. couchianus* (Yang et al. 2009).

Future Research Topics

With the expanding use of aquarium fishes as research models, new mutants, transgenic individuals, strains, and lines are being continually created. To protect and maintain this burgeoning number of valuable individuals, strains and lines, it is essential to develop a germplasm repository program with gamete and embryo cryopreservation. Sperm cryopreservation will be the main focus based on current technologies and approaches until the development of protocols for egg and embryo cryopreservation in fish. At present, protocols for sperm cryopreservation are available for use with zebrafish, medaka, and *Xiphophorus* fishes, and live offspring have been produced with cryopreserved sperm. Future research needs include the following topics.

Standardization of the Procedures for Sperm Cryopreservation

To enable application of protocols developed for sperm cryopreservation across different laboratories, procedural standardization is necessary, especially for determination and choice of sperm concentration, sample packaging, and labeling. Standardization is also necessary for terminology (e.g., strict definition of terms such as fertilization and motility) and in defining the essential parameters necessary for complete reporting of results. Sperm concentration is an extremely important factor in sample preparation for cryopreservation, and can directly influence results (Dong et al. 2007). Because most studies of aquatic species cryopreservation do not control or report sperm concentrations, it is likely that this is the single largest uncontrolled variable in this research area (Tiersch et al. 2007). Also, due to the limited sperm availability in aquarium fishes, maximized use of cryopreserved samples for strain or line recovery requires control of sperm concentration and determination of suitable sample loading in each container (e.g., French straw). Hemocytometer counts and spectrophotometry are commonly used methods to measure sperm concentration. For aquarium fishes, specialized spectrophotometers with a 2-µl sample size are desirable to avoid sample waste (Tan et al. 2010).

Evaluation of Gamete Quality

Considerable variation in characteristics of post-thaw sperm is generally the rule in most species studied (Mazur et al. 2008), and has been found in zebrafish (Yang et al. 2007a), and *X. couchianus* (Yang et al. 2009). The reasons for this variability, for example a range in post-thaw sperm fertility of from 5 to 81% in samples with the same initial motility in zebrafish (Yang et al. 2007a), have not been identified. A more comprehensive understanding of sperm quality needs to be obtained for assessing within-species variation in the susceptibility of spermatozoa to damage during cryopreservation, including factors such as membrane integrity and mitochondria function.

A genetic basis for variation in post-thaw semen viability has been suggested (Thurston et al. 2002), and it has been proposed that certain molecular markers could be identified to link with the genes influencing this variation. Currently, estimation of sperm quality before cryopreservation provides an opportunity to predict the outcome of sperm cryopreservation, but it will be necessary to develop a functional and molecular understanding of the factors that influence sperm cryopreservation. Sperm quality estimation could include: analysis of the relationship of sperm performance with body condition and nutrition, the use of flow cytometry for measuring membrane integrity and mitochondrial integrity, the use of computer-assisted

sperm analysis (Cosson 2004) for quantifying motility characteristics, and detection of changes in protein profiles in sperm after cryopreservation for predictive biomarkers.

Establishment of a Working Repository System for Cryopreserved Sperm

After cryopreservation, frozen samples stored in liquid nitrogen require permanent and clear identification. The use of plastic straws for packaging of sperm samples offers the advantage of permanent labeling by direct printing and the use of automated barcode readers. A labeling and coding system for each cryopreserved sample needs to be developed, including information on biology, genetics, intellectual property rights, source and ownership, sample collection, and handling. Integrated databases need to be developed with the existing genetic databases for these fishes, inventory procedures need be established to allow easy access to cryopreserved samples from a specific species and strain, and biosecurity procedures need be established to minimize or prevent transfer of pathogenic contaminants with cryopreserved samples (Tiersch and Jenkins 2003).

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Appendix

1. BSMIS (Morris et al. 2003):
   75 mM NaCl, 70 mM KCl, 2mM $CaCl_2$, 1mM $MgSO_4$ and 20 mM Tris, pH 8.0, store at 4 ºC.
2. BSS (balanced salt solution) (Iwamatsu 1983):
   Solution A: 0.111 M NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$-$7H_2O$
   Solution B: 0.6 M $NaHCO_3$. Working solution: adjust solution A with solution B to bring the pH to 7.4
3. Ginsberg buffer (Ginsburg 1963):
   0.111 M NaCl, 3.4 mM KCl, 2.7 mM $CaCl_2$-$2H_2O$, 2.4 mM $NaHCO_3$. Note: the order of addition is important to prevent precipitation. Freezing medium (see: www.zfin.org): 9 mL Ginsburg buffer, 1 mL methanol, and 1.5 g powdered skim milk. The order of ingredients is important to prevent precipitation of the milk, and this medium needs to be used within 3 hr.
4. HBSS (Hanks' balanced salt solution) (Hanks 1975, Tiersch et al. 1994):
   0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.8.

New Chapter

Cryopreservation of Striped Bass Spermatozoa

L. Curry Woods III

Introduction

This chapter on the cryopreservation of spermatozoa of wild and captive striped bass *Morone saxatilis* is a sequel to the information provided by Brown and Brown (2000) for striped bass and white bass *Morone chrysops* in the first edition of the book *Cryopreservation in Aquatic Species* (Tiersch and Mazik 2000). The reader is encouraged to consult the original chapter for a thorough description of semen collection methods for these fish. Similarly, if the reader is interested in moronid reproduction, or spermatogenesis and spermiation in striped and white bass, I would direct them to the review by Sullivan et al. (1997). Considerable interest exists in developing an effective, commercially relevant cryopreservation protocol for striped bass semen due to the growing worldwide industry for striped bass aquaculture (Woods 2005). Interest in efficacious methods to store germplasm is especially relevant for this cultured fish as the current industry utilizes striped bass spermatozoa for *in vitro* fertilization of white bass eggs to produce hybrid bass (Woods 2005, Jobling et al. 2009). This chapter will therefore focus primarily on the recent scientific information for the cryopreservation of striped bass semen.

Extenders and Cryomedia

The ultrastructure, fertilization capacity, and motility of fresh and cryopreserved spermatozoa were examined by use of six cryomedia with various levels of dimethyl sulfoxide (DMSO) and glycine (He and Woods 2004a). The plasma membranes of spermatozoa were best protected with the cryomedia containing DMSO and glycine. The highest percentage of fertilized striped bass eggs, equivalent to 90% of fresh semen controls, was also obtained using cryomedia containing 75 mM glycine and with a final DMSO concentration of 7.5%. This suggested that glycine can improve fertilization capacity of spermatozoa with DMSO as the cryoprotectant. In addition, post-thaw motility of striped bass sperm frozen using DMSO was enhanced with glycine and this effect was independent of the DMSO concentration (He and Woods 2003a). The mechanism by which glycine improves fertilization capacity of cryopreserved spermatozoa is not clear. Ultrastructural analysis did not demonstrate that glycine provided additional protection to the plasma membrane. Glycine is thought to bind to the membrane of spermatozoa, reducing the speed of DMSO permeation and potentially minimizing osmotic shock or toxicity to the cell.

To build upon this cryomedia developed with DMSO and glycine, plasma membrane integrity and mitochondrial function were evaluated in cryopreserved sperm (He and Woods 2004b). Prior to freezing, no concentration of DMSO evaluated (2.5 - 10% final, v/v) was found to significantly ($P > 0.05$) damage the integrity of plasma membranes after sperm were exposed for as long as 10 min. The ultrastructure of post-thaw plasma membranes, evaluated by fluorescent staining and by scanning electron microscopy, indicated that with increasing DMSO concentration, membranes were better protected, and 10% DMSO had the highest percentage of sperm with intact membranes. However, sperm mitochondrial function decreased significantly with increasing DMSO concentration. The addition of glycine to the cryomedia significantly

Woods III, L.C. 2011. Cryopreservation of Striped Bass Spermatozoa. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 455-458.

increased the percentage of sperm with post-thaw functional mitochondria and ATP content. However glycine did not provide protection to post-thaw membrane integrity. The highest percentage of sperm with intact membranes and functional mitochondria was obtained with 7.5% DMSO and 75 mM glycine.

Motility of white bass and striped bass spermatozoa is initiated in fresh water, where both species spawn (Woods 2005). Striped bass sperm has also been shown to activate in various experimental media with osmoalities of 500 mOsmol/kg or less (He and Woods 2003b). The broad range that can activate sperm may be explained by the fact that striped bass is an anadromous species, which migrates from saltwater to fresh water to spawn. To adapt to environmental conditions with such a large variation in osmolality, striped bass may have developed a mechanism by which activation of sperm motility could be initiated in fresh or brackish waters (He et al. 2004). While the activation mechanism is not clear, it has been demonstrated that K^+, H^+, Ca^{2+}, Mg^{2+}, cAMP, and osmolality are not the key factor that maintains sperm quiescent in seminal plasma (He et al. 2004). The duration of sperm motility lasts 30-60 sec normally (Jenkins-Keeran and Woods 2002), so available energy becomes a critical factors to initiate and keep sperm motile. Equilibration time is important for cryopreservation of striped bass sperm when using DMSO as a cryoprotectant. The shortest equilibration time examined, 10 min, showed the best pre-freezing and post-thaw motilities.

Hyperosmotic media (600 mOsm/kg) have been used to block the activation of striped bass spermatozoa, and to prohibit activation prior to cryopreservation (He and Woods 2003b). However, this may only be used for short periods ($\leq$ 10 min) and is therefore an impractical extender to use when hr or d of storage or shipment are required. Because the effects of hyperosmolality must be considered in the design of extenders, a unique approach to assess the energetic status of striped bass spermatozoa for the relatively short periods of storage, as would be expected between collection and cryopreservation, was recently developed (Guthrie et al. 2008). The energy status of testicular spermatozoa was determined after being incubated in a TRIS free base-NaCl medium (pH 8), adjusted to iso-osmotic (300 mOsmol/kg) or hyperosmotic conditions (600 mOsmol/kg) with NaCl. Flow cytometry, with the mitochondrial probe 5, 50, 6, 60-tetrachloro-1, 10, 3, 30-tetraethylbenzimidazolyl-carbocyanine iodide was used to determine the mitochondrial transmembrane energy potential in conjuction with a luciferin-luciferase assay to measure total cellular ATP. Spermatozoa maintained on ice were equally viable (at >95%) for the 300 and 600 mOsmol/kg solutions, for as long as 80 min. Of note, the viability of sperm maintained on ice in fresh water (27 mOsm/kg) decreased to 67% after 5 min, and to 4% after 25 min. However, after 20 min, 80% of the spermatozoa maintained a high mitochondrial potential at 300 mOsmol/kg solution compared to 50% when held in the 600 mOsmol/kg solution. Although viable, the sperm ATP content after 20 min of equilibration was 2.9 pmol/10^6 cells in spermatozoa incubated in the 300 mOsmol/kg solution, compared to 0.8 pmol/10^6 cells at 600 mOsmol/kg, demonstrating the high energetic ‘cost’ of storage at a high osmolality (600 mOsmol/kg), even when motility is prohibited (Guthrie et al. 2008).

Cryopreservation Protocol Summary

The following protocol is a synthesis of the above-mentioned research (Woods et al. 2008) and has demonstrated excellent post-thaw results for striped bass spermatozoa including cell viability, functional mitochondria, and preservation of ultrastructure. Fresh semen is diluted

with the striped bass extender (Table 1) in a 1:1 ratio and placed over crushed ice for 10 min to allow the cells to acclimate to the high osmolality.

Table 1. Components of the extender and cryomedia used for striped bass.

Component	Extender	Cryomedia
KCl	40 mg	40 mg
NaCl	1400 mg	1400 mg
$NaHCO_3$	200 mg	200 mg
Dimethyl sulfoxide*	- -	15 mL
Glucose	100 mg	100 mg
Glycine	75 mM	75 mM
Deionized water	100 mL	85 mL
pH (adjusted by adding 1 N NaOH)	7.6	7.6

*Sigma-Aldrich, St. Louis, Missouri, USA (D8418).

The extended semen is diluted 1:1 with cryomedia containing 15% DMSO. This provides a final DMSO concentration of 7.5% and a final semen:extender dilution of 1:3. Equilibration time prior to freezing, should be $\leq$ 10 min. During this time, aliquots (150 ul) of the cryoprotected semen should be pipetted into chilled 500-ul French straws. Normally two people can load, heat seal, and place 80 straws on freezing racks in the controlled-rate freezer. The cooling rate is at -40 ºC/min until reaching -120 ºC when straws are plunged into liquid nitrogen. Frozen straws are thawed in a 35 ºC water bath for 8 sec. The fertilization rate of striped bass semen frozen and stored for almost 1 yr, using this protocol, has been tested in commercial trials (He and Woods 2004a) and provides fertilization rates with striped bass eggs equivalent to 90% of fresh controls. For fertilization, post-thaw semen is immediately placed directly onto eggs followed by the addition of fresh water (1:5 ratio of semen:water). An effective sperm-to-egg ratio is approximately 4×10^6:1. No difference in the fertilization percentage was observed between the control and extender containing 7.5% DMSO with 75 mM glycine. Glycine, in this study at a commercial aquaculture facility, had a significant beneficial effect on fertilization when added to cryomedia containing DMSO. Future research to identify the factors that maintain striped bass sperm immotile (preferably in isotonic solution) is needed.

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Cryopreservation of Sperm of Some Marine Fishes

Jin-Chywan Gwo

Introduction

Recent increases in the demand for seafood have led to a growing interest in the culture of several marine fish species. Cryopreservation of fish sperm would facilitate reliable, long-distance shipping of large quantities of desirable samples; ready availability of sperm for hybridization and genetic studies, and preservation of sperm from exceptional individuals and endangered species for future use. For example, some cultured fishes of the family Serranidae (the groupers) are protogynous hermaphrodites, and functional males are reported to mature at 10 yr of age, when reaching 20 Kg or more. Due to their large size, the source of male broodstock is limited and collection of sperm is a problem. Many fishes of the family Sparidae (the seabreams) are protandrous hermaphrodites and self-fertilization using cryopreserved sperm is possible. Cryopreservation of sperm could reduce the number of males needed in the hatchery, minimize handling stress through less frequent stripping, facilitate genetic understanding and quickly lead to the development of inbred lines. For example, frozen sperm of crimson sea bream *Evynnis japonica* can be used to fertilize female red sea bream *Pagrus major* and to produce hybrids with increased growth rate and desirable body color (Kurokura et al. 1984). Cryopreservation of sperm has been used for selective breeding in the gilthead sea bream *Sparus aurata* (Happe and Zohar 1986). Cryopreservation of sperm of sex-reversed gynogenetic female hirame *Paralichthys olivaceus* has been used in the production of feminized seedstocks (Tabata and Mizuta 1997).

To date, sperm of about 200 fish species have been cryopreserved and over 300 studies have been published following the discovery that glycerol could act as a cryoprotectant for fowl sperm in 1949 (Billard et al. 1995). The majority of studies are related to cultivated salmonids of aquacultural importance (Scott and Baynes 1980). In contrast, the literature on cryopreservation of marine fish sperm is fairly limited. Few reviews have emphasized the cryopreservation of marine fish sperm, although the first report of successful cryopreservation of fish sperm was a marine fish, the Atlantic herring *Clupea harengus* (Blaxter 1953). Since this work, cryopreservation protocols have been developed for sperm of over 40 species of marine fishes. These include representatives of the orders Clupeiformes, Gadiformes, Perciformes, Pleuronectiformes and Tetraodontiformes. Attempts at sperm cryopreservation have been more successful and consistent in marine fish than those made in freshwater fish.

Many published cryopreservation techniques for marine fish sperm are incomplete or remain unclear. No differences in fertilization rate between thawed and fresh sperm were reported for the American plaice *Pleuronectes platessoides* (Pullin 1972), Atlantic cod *Gadus morhua* (Mounib 1978) or Atlantic halibut *Hippoglossus hippoglossus* (Bolla et al. 1987); however, neither egg quantity nor the dilution ratio of sperm at artificial insemination were described. Also, various factors such as freezing and thawing rates have not been measured or reported and the effects of cryoprotectant concentration, equilibration time and freezing and thawing methods on post-thaw fertility have not been rigorously tested. Furthermore, due to the difficulty in obtaining good quality eggs with induced spawning techniques, fertility evaluation tests frequently were not conducted and only motility of thawed sperm was reported (Doi et al. 1982, Hara et al. 1982, Withler and Lim 1982, Leung 1987). Thus, most of the work is empirical,

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based on a trial and error approach, leading to diverse results and universal protocols do not exist.

Biology of Marine Fish

Initiation of Sperm Motility in Marine Fish

Sperm of oviparous fishes are normally immotile in the male reproductive organs and in the semen (Stoss 1983, Morisawa 1994). Upon discharge into the natural environment, sperm begin vigorous flagellatory movements and metabolic rate is greatly enhanced (Morisawa and Suzuki 1980, Inoda et al. 1988, Gosh 1989, Morisawa and Morisawa 1990). The initiation of sperm motility is known to be triggered by the difference between the environment within the male and that encountered after discharge. A decrease in external osmolality is the signal for the initiation of sperm motility in freshwater cyprinid fishes (Morisawa and Morisawa 1990, Takai and Morisawa 1995), while hyperosmolality has been proposed to be the trigger of initiation of sperm motility in marine fishes (Oda and Morisawa 1993, Takai and Morisawa 1995). The presence of ions is not necessary for the initiation of sperm motility in Atlantic halibut, marine puffer *Takifuku niphobles,* yellowtail flounder *Pleuronectes ferrugineus*, turbot *Scophthalmus maximus*, gilthead seabream, and grey mullet *Mugil cephalus* (Suquet et al. 1994). Shrinkage or swelling of sperm cells by change of the environmental osmolality occurs and causes increase or decrease in intracellular potassium which triggers the initiation of flagellular movement (Takai and Morisawa 1995). However, factors other than osmolality also are involved in initiating sperm motility in some marine teleosts. Sperm of the Pacific herring *Clupea palasii* are immotile in seawater and are induced to vigorous motility when they reach the egg surface. The motility-initiating factor (a non-diffusible 105 kDa glycoprotein) has been localized to the region of the micropyle (Yanagimachi et al. 1992, Griffin et al. 1996). In contrast, a sperm-activating substance (an acidic 8.1 kDa peptide) obtained from water in which eggs have been incubated, has also been reported to enhance the sperm motility of Pacific herring (Oda and Morisawa 1993).

A decrease in external potassium concentration is essential for the initiation of Salmonid sperm motility (Stoss 1983, Morisawa 1985) and an increase in intracellular calcium and potassium concentrations have recently been proposed to trigger the initiation of sperm motility in marine teleosts (Yanagimachi et al. 1992, Oda and Morisawa 1993, Takai and Morisawa 1995). The presence of membrane receptors for 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) have been demonstrated for sperm of Atlantic coaker *Micropogonus undulus* (Ghosh and Thomas 1995). The identification of progestin receptors on marine teleost sperm suggests that the hormonal regulation of sperm activation may be similar to that in mammals. It also raises the possibility that 20β-S may be involved in calcium influx and the activation of sperm motility. Simple pH has also been shown to be an activating factor in sperm of Atlantic halibut (Billard et al.1993), marine puffer, yellowtail flounder and ocean pout *Macrozoarces americanus* (Wang and Crim 1997). The sperm of ocean pout, an internally fertilizing marine fish, show high motility in NaCl solutions of 300 to 400 mOsmol/Kg, but were immobilized instantly upon the addition of seawater (Yao and Crim 1995, Wang and Crim 1997). These sperm were motile before leaving the reproductive tract and the motility lasted for d (Yao and Crim 1995, Wang and Crim 1997).

The Effect of Osmolality on the Ultrastructure of Marine Fish Sperm

In spite of the diverse taxonomic relationships of the families Sparidae, Serranidae and Sciaenidae, sperm of these fishes demonstrated a common ultrastructural change upon hypo-osmotic shock and after activation in artificial sea water (Gwo 1995). The hypo-osmotic medium (distilled water) induced a dramatic morphological distortion in sperm accompanied by plasma membrane and mitochondrial disruption (Gwo 1995). Fish sperm exhibit species-specific resistance to changes in osmotic pressure. Sperm of common carp *Cyprinus carpio* were disrupted in distilled water (Gwo 1982) and fresh water (Morisawa et al. 1983b) although in rainbow trout *Onchorhynchus mykiss* and some marine teleosts, sperm head chromatin retained its original shape even in distilled water (Gwo 1982, 1995, Morisawa et al. 1983a). The composition of sperm phospholipids in marine and freshwater fishes differs significantly (Drokin 1993). Phosphatidylcholine, which protects sperm from both osmotic and cold shock, predominates over other lipids in the sperm of marine fishes compared with those of freshwater fishes (Drokin 1993). It is probable that the differences in sperm resistance to osmotic shock among fish species is related to the sperm cell membrane properties.

Following activation of the sperm of marine fishes in artificial sea water, the mitochondria were shrunken and disappeared at the end of motility duration, with no obvious change in other ultrastructure (Gwo 1995). This observation suggests that marine fish sperm motility is closely related to the existence of mitochondria in the mid-piece.

Although fish sperm are capable of using external energy sources (Stoss 1983, Gosh 1989, Billard and Cosson 1990), it is unlikely that they would be able to use exogenous substrates for energy production, since virtually no nutrients are present in the fertilization environment. Previous studies indicate that oviparous fish sperm obtain energy for movement through the oxidation of endogenous substrates (Stoss 1983, Christen et al. 1987, Inoda et al. 1988, Billard and Cosson 1990, Lahnsteiner et al. 1992). The mitochondria are the reservoirs of the endogenous energy source for the motility of oviparous marine fish sperm. Sperm of viviparous fishes may also metabolize extracellular glucose.

The Motility Pattern of Marine Fish Sperm

The percentage of motile sperm, swimming velocity and beat frequency of Atlantic halibut and turbot sperm decreased soon after dilution and remained stable over the first 50 sec and then dropped suddenly, although most sperm continued to vibrate (Billard et al. 1993, Chauvaud et al. 1995). The motility pattern was different from those of common carp and rainbow trout in which the flagellar beat frequencies and the percentages of motile sperm decreased steadily after activation. The duration of motility and the decline of the flagellar beat frequency were due to exhaustion of endogenous stores of ATP. Rapid exhaustion of ATP and the inability of the mitochondria to sustain the high energy demand during motility was found to cause the short duration of rainbow trout sperm motility (Christen et al. 1987). In contrast, it has been suggested that ATP was not pre-accumulated, but rather was elaborated by the mitochondria during the motility period in sperm of Atlantic halibut and turbot (Billard et al. 1993). The initiation of Atlantic halibut sperm motility included a concomitant stimulation of the process of respiration. Alteration of the sperm membrane, exhaustion of mid-piece substrates, and the lack of a shuttle molecule to transport ATP to the distal part of the flagellum might be the reasons for the sudden drop in beat frequency (Billard et al. 1995).

Cryopreservation

The procedures for freezing of marine fish sperm and the possible variables which can influence sperm fertilizing ability include: 1) extenders; 2) dilution ratios; 3) cryoprotectants; 4) concentration of cryoprotectants; 5) equilibration time; 6) freezing rate; 7) freezing method; 8) thawing temperature; 9) sperm quality; 10) egg quality, and 11) sperm-to-egg ratio.

Extenders

Development of a suitable extender is the first step in cryopreservation of fish sperm. Graybill and Horton (1969) defined the extender as "a solution of salts, sometimes including organic compounds, which helps maintain the viability of cell during refrigeration." According to Graham (1978), an ideal extender for sperm should: "1) maintain proper electrolytic balance and osmotic pressure within the cells to ensure minimal salt effects; 2) contain constituents with metal-complexing properties that can effectively bind heavy metals which are harmful to spermatozoa; 3) have a pack of between 6 and 8 to provide buffering capacity against deleterious effects of shifts in the pH as metabolic wastes accumulate; 4) be stable, resisting enzymatic and non-enzymatic degradation; 5) contain antibiotics that inhibit bacterial contamination of the sample; 6) allow for the substantial increase of sperm volume to maximize insemination potential, and 7) provide an environment suitable for the normal metabolic activities of the spermatozoa."

For fish sperm, an extender that does not initiate the motility of spermatozoa seems to be the most important requirement. There have been a number of extenders developed for marine fish sperm (Table 1). For example, Mounib's sodium chloride medium was used for Atlantic cod (Mounib et al. 1968), American plaice (Pullin 1972) and Atlantic halibut (Bolla et al. 1987). Fish blood serum was used for milkfish *Chanos chanos* (Hara et al. 1982) and diluted sea water was used for Atlantic herring (Blaxter 1953) and yellowfin bream *Acanthopagrus australis* (Thorogood and Blackshaw 1992). Simple extenders of glucose, sodium citrate or sodium chloride have been used successfully in the cryopreservation of marine fish spermatozoa (Chao et al. 1992, Gwo 1982, Hara et al. 1982, Miyaki and Dotsu 1987, Gwo et al. 1991, Gwo 1993). Organic materials, including bovine serum albumin, lecithin, protein and egg yolk are widely used for freezing of Salmonid sperm. However, no improvement in fertilization rates was obtained with the sodium chloride extender by addition of bovine serum albumin, egg yolk or skim milk powder in Atlantic croaker (Gwo et al. 1991), barramundi *Lates calcarifer* (Palmer et al. 1993) and turbot (Dreanno et al. 1997). Depending on the cryoprotectant, the addition of egg yolk induced either positive or negative effects on motility of thawed barramundi sperm (Leung 1987). Species-specific differences were found between the fertilization capacity of frozen spermatozoa from yellowfin seabream *Acanthopagrus latus* and Atlantic croaker when using the same extender (sodium citrate) (Gwo 1995). Other extenders reported for marine fishes proved unsuitable for Atlantic croaker; among these were BDCSB4 and Mounib's sodium chloride medium (Gwo et al. 1991).

Dilution Ratio

In sperm of sea bass *Dicentrarchus labrax* and gilthead seabream, a decrease in fertility occurred when dilution exceeded one-half (Billard 1978a). The dilution ratio of gilthead seabream sperm was suggested to be in the range of 2 to 10 (Chambeyron and Zohar 1990). No significant effect on post-thaw motility was noted when turbot sperm were diluted at ratios of 1:1, 1:2, 1:4 and 1:9 (Dreanno et al. 1997). Post-thaw fertility of Atlantic croaker sperm was

Table 1. Composition of extenders used in the cropreservation of marine fish sperm. Ingredients are listed as g/L.

	ASPOP	ASPR	DCS B4	DCS D2	FW FR	G1	G2	HBSS	IHR	KK	MO	MMM	MNM	MTR	OH-129	OH-164	OH-189	OH-251	Plaice Ringer
NaCl	10.7	8.01	19.5	9.5	7.5	--	10	8.01	12.05	--	--	--	19.4	7.25	7.3	7.3	7.3	8.5	8.22
KCl	--	0.82	--	--	0.2	--	--	0.4	0.54	--	--	--	--	0.38	0.38	0.38	0.38	--	0.39
KOH	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
$CaCl_2$	--	--	--	--	0.4	--	--	--	0.24	--	--	--	--	--	0.23	0.23	0.23	--	--
$CaCl_2$-$2H_2O$	--	--	0.25	--	--	--	--	0.16	--	--	--	--		0.24	--	--	--	--	0.72
$NaHCO_3$	--	--	--	--	--	--	--	0.35	0.08	--	--	--	6.2	0.01	5	7.5	5	5	0.2
$CaCO_3$	0.15	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
$MgSO_4$-$7H_2O$	0.18	--	0.25	--	--	--	--	0.2	--	--	--	--		0.27	0.23	0.23	0.23	--	--
$MgCl_2$-$6H_2O$	--	--	--	--	--	--	--	--	0.65	--	--	--	--	--	--	--	--	--	0.23
NaH_2PO4-H_2O	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.41	0.41	0.41	--	--
NaH_2PO_4-$2H_2O$	--	--	--	--	--	--	--	--	--	--	--	--	--	0.41	--	--	--	--	0.28
NaH_2PO_4-$7H_2O$	--	0.98	--	--	--	--		0.12	--	--	--	--	--	--	--	--	--	--	--
Na_2HPO_4	--	--	--	--	--	--	--	0.04	--	--	--	--	--	--	--	--	--	--	--
KH_2PO_4	--	--	--	--	--	--	--	0.06	--	--	--	--	--	--	--	--	--	--	--
$KHCO_3$	1.03	--	--	--	--	--	--	--	--	--	10.0	10.0	--	--	--	--	--	--	--
Glutathione	--	--	--	--	--	--	--	--	--	--	2.0	2.0	--	--	--	--	--	--	--
Sucrose	--	--	--	--	--	--	--	--	--	--	42.7	42.7	--	--	--	--	--	--	--
Fructose	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1.0	1.0	5.0	--	--
Glycine	--	--	6.25	6.25	--	--	--	--	--	--	--	--	2.2	--	--	--	--	--	--

Glucose	0.05	--	--	--	--	5.6	--	0.35	--	--	--	--	--	1.01	--	--	--	--	1.0

(Table 1. Continued)

	ASPOP	ASPR	DCS B4	DCS D2	FW FR	G1	G2	HBSS	IHR	KK	MO	MMM	MNM	MTR	OH-129	OH-164	OH-189	OH-251	Plaice Ringer
Lecithin	--	7.5	--	--	--	--	--	--	--	--	--	--	--	--	5.0	7.5	7.5	15.0	--
Mannitol	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2.5	5.0	--	--
Sodium Citrate	--	--	--	--	--	--	--	--	--	25.8	--	--	--	--	--	--	--	--	--
TRIS	--	--	2.4	2.4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
BSA	--	--	--	--	--	--	--	--	--	--	--	10.0	--	--	--	--	--	--	--
pH	--	--	8.5	7.5	7.0-7.3	--	--	--	7.8	--	--	7.8	--	--	7.0	--	--	--	--
mOsmol	--	--	720	760	--	--	--	--		--	--	310	--	--	--	--	--	--	--

ASPOP, (Artificial seminal plasma of ocean pout), Yao et al. (1995)
ASPR, (Atlantic salmon dilution 1; Plaice Ringer), Richardson et al. (1995)
DCS B4, Billard (1984)
DCS D2, Billard (1984)
FWFR, (Freshwater fish Ringer), Tabata and Mizuta (1997)
G1, Gwo et al. (1991)
G2, Gwo et al. (1991)
HBSS, (Hanks' balanced salt solution), Tiersch et al. (1994)
IHR, (isotonic herring Ringer), Pillai et al. (1994)
KK, Kurokura et al. (1986)
MMM, (Modified Mounib medium), Dreanno et al. (1997)
MNM, (Mounib's NaCl medium), Mounib et al. (1968)
MO, (Mounib's medium), Mounib (1978)
MTR, (Marine teleost Ringer), Palmer et al. (1993)
OH-129, Ott and Horton (1971)
OH-164, Ott and Horton (1971)
OH-189, Ott and Horton (1971)
OH-251, Ott and Horton (1971)
Plaice Ringer, Richardson et al. (1995)

best at dilution ratios of 1:5 to 1:20 (Gwo et al. 1991). Some type of dilution effect may take place that exhausts fish sperm at higher dilution ratios and thus it appears to be beneficial to freeze marine fish sperm with low dilution ratios.

Cryoprotectants

Cryoprotectants are essential for the survival of spermatozoa during cryopreservation. The major properties of cryoprotectant are to bind water, reduce ice crystal formation and to bind electrolytes and prevent them from concentrating in the residual unfrozen solution, thereby decreasing the freezing point of intracellular fluid during freezing. A number of compounds have been identified as possessing cryoprotective qualities. They are generally considered to fall into two main groups: those which penetrate the cell (e.g. methanol, dimethyl sulfoxide, glycerol and other polyols) and those which are non-penetrating (various sugars and proteins). Extenders containing sugars (glucose or sucrose) have been advantageous in freezing sperm of bluefin tuna *Thunnus thynnus*, Atlantic croaker, black grouper *Epinephelus malabaricus* and marine puffer (Doi et al. 1982, Gwo 1982, Chao et al. 1986, Gwo et al. 1991, Gwo 1993). Non-penetrating cryoprotectants presumably act osmotically to promote cell dehydration during freezing. The cryoprotective effect of sugars is assumed to be related to their membrane-stabilizing activity. Sugars such as sucrose and trehalose have been shown to stabilize liposomal membranes during freezing (Quinn 1985, Anchordoguy et al. 1987). In addition, sugars are less toxic to spermatozoa than are many cryoprotectants, which may prove beneficial for cryopreservation. A synergistic effect between the combination of glucose and dimethyl sulfoxide (DMSO) in the diluent was found when freezing yellowfin sea bream sperm. The combination of 300 mM glucose plus DMSO yielded a higher fertilization rate after thawing than did 300 mM glucose (Gwo 1994). Similar results also have been observed for sperm of Atlantic croaker and marine puffer (Gwo et al. 1991, 1993a, 1993b).

For cryopreservation of fish sperm, DMSO has found widespread acceptance (Stoss 1983, Rana et al. 1995). Although DMSO and glycerol at concentrations of between 5% and 20% (v/v) have been widely used in cryopreservation of marine fish sperm, DMSO was superior to glycerol for sperm of yellowfin sea bream, black grouper, barramudi and turbot (Gwo 1993, 1994, Palmer et al. 1993, Dreanno et al. 1997). There are indications that the presence of glycerol lowers the fertilizing ability of sperm in several fish species. For example, glycerol caused a total loss of motility of grouper *E. tauvina* sperm (Withler and Lim 1982) and appeared to be toxic to sperm of Pacific herring and turbot (Pillai et al. 1994, Dreanno et al. 1997). To minimize cryoprotectant toxicity, the equilibration time is usually kept to a minimum. No information on the degree and speed of glycerol penetration into fish spermatozoa is available; however, fish spermatozoa are sufficiently small that penetration usually occurs rapidly (Stoss 1983). Glycerol is osmotically active and was slow in permeating sperm membranes of yellowfin seabream (Gwo 1994) and barramundi (Palmer et al. 1993). A step-wise addition of glycerol was recommended to lessen its contribution to the osmolality of the extender and hence to increase the survival of rainbow trout spermatozoa (Erdahl and Graham 1980). Glycerol was also reported to have a cryoprotective effect at equilibration times longer than 20 min, while DMSO was effective at shorter equilibration times (Doi et al. 1982). Yellowfin sea bream sperm, diluted in 10% glycerol, would not become motile when equilibration times were 60 min or longer (Gwo 1994).

Various mixtures of extender and cryoprotectant have been tested to find a combination that prevents activation of spermatozoa during dilution prior to freezing, thus preserving energy

needed for fertilization and for the protection of sperm morphology (Billard 1978a, Billard and Cosson 1990). Intensive motility was observed before freezing as soon as yellowfin sea bream sperm were diluted with glycerol (Gwo 1994). Upon thawing, the yellowfin sea bream sperm regained spontaneous motility without further addition of seawater. Because of the short duration (60 to 70 sec) of progressive motility of yellowfin sea bream sperm, glycerol, with its direct osmotic effect on sperm cells, could exhaust the energy needed for fertilization. Use of high sperm concentration appeared to circumvent this problem. Fertility for black porgy *A. schlegeli* sperm was obtained by using a low sperm dilution ratio (1:1) with extender plus cryoprotectant (Chao et al. 1986).

Propylene glycol and ethylene glycol are used as cryoprotectants for their ability to rapidly penetrate cell structures and for their limited toxicity, thereby producing less osmotic shock. Propylene glycol has been demonstrated as an effective cryoprotectant for sperm of yellowtail flounder and Atlantic halibut (Billard et al. 1993, Richardson et al. 1995) and was more suitable than DMSO and glycerol for Atlantic halibut sperm (Billard et al. 1993). Motility of fresh and thawed sperm of yellowfin sea bream was higher when 10% glycerol was replaced by either 10% propylene glycerol or 10% ethylene glycerol (Gwo 1994). The molecular weights of these cryoprotectants are lower than that of glycerol, which may explain the better results achieved using them for yellowfin sea bream. However, ethylene glycol appeared to be toxic for sperm of turbot (Dreanno et al. 1997) and Pacific herring (Pillai et al. 1994). Poor results with methanol were reported for marine teleost sperm such as barramundi, Atlantic croaker, black grouper, yellowfin sea bream and turbot (Gwo et al. 1991, Gwo 1993, 1994, Palmer et al. 1993, Dreanno et al. 1997).

Concentration of Cryoprotectant and Equilibrium Time

In marine fishes, best results have been obtained with 10 to 20% DMSO (e.g., Wayman et al. 1997). A short equilibrium time (5 min) for Atlantic croaker sperm in the extender and cryoprotectant prior to freezing produced a significantly higher fertility rate than did an equilibrium time of 60 min (Gwo et al. 1991). Increasing DMSO concentration and simultaneously increasing equilibration time resulted in decreased fertility. Best results were obtained when a 5-min equilibration was used with 20% DMSO (Gwo et al. 1991). Prolonged equilibration was detrimental to Atlantic halibut sperm and minimal equilibration resulted in the best fertility (Billard et al. 1993). A similar finding was made for turbot (Dreanno et al. 1997), gilthead seabream and sea bass (Billard 1978b). Optimal concentrations of cryoprotectants could be influenced by the extenders used, dilution ratio, freezing and thawing methods or species. For example, there were no differences in post-thaw fertility when 15 and 20% concentrations of DMSO were used to preserve Atlantic croaker spermatozoa (Gwo et al. 1991), although DMSO concentrations of 5% gave the best post-thaw motility for barramundi sperm (Leung 1987).

Freezing Rates

The range of optimum freezing rates for Atlantic croaker sperm was from 10 to 150 °C per min (Gwo et al. 1991). The estimated optimal freezing rates for sperm of black grouper, yellowfin sea bream and turbot were similar to the rates reported for Atlantic croaker (Gwo 1993, 1994, Dreanno et al. 1997). Freezing rates of between 21 and 119 °C per min were favorable for grey mullet sperm (Chao 1982). No optimum range of freezing rate was found for crimson sea bream sperm between 20 and 160 °C per min (Kurokura et al. 1986). However, the optimum freezing rate for gilthead seabream and sea bass was 10 to 20 °C per min (Billard

1978b), with slower and faster freezing rates being less favorable. Fertilization has been reported using sperm of Atlantic cod (Mounib 1978) and Pacific herring (Pillai et al. 1994) that were frozen by immersion in liquid nitrogen. In Atlantic halibut, freezing has been carried out by pelleting suspended sperm on dry ice (Bolla et al. 1987, Billard et al. 1993). The freezing rate was estimated as 20 °C to 30 °C per min. The susceptibility of fish sperm to freezing damage can vary among species as demonstrated in mammals where the amount of damage associated with cold shock is species dependent (Watson 1981).

Freezing Methods

The two-step freezing method has been applied successfully to spermatozoa of a number of mammalian species (Ashwood-Smith and Farrant 1980). With this method, freezing occurs during the initial period of rapid cooling to a constant holding temperature. After maintaining the sample at this temperature for some time, it is cooled to the final storage temperature (-196 °C). It is assumed that the cells are minimally affected by the potentially damaging hypertonic solution. Shrinkage occurs at the holding temperature, and it is this shrinkage that reduces the probability of intracellular ice formation during rapid cooling to -196 °C. This method has two practical advantages. It is simple to carry out and requires no equipment for controlled cooling rates. There was no fertility for Atlantic croaker sperm when the first step temperatures in the two-step method were above 0 °C or below -90 °C (Gwo et al. 1991). The best fertility rate for Atlantic croaker sperm was obtained with a first-step temperature of -50 °C.

Thawing Temperatures

Thawing temperature can have profound effects on sperm viability after freezing. No significant difference in fertility was found between two thawing temperatures (25 °C and 50 °C) for Atlantic croaker sperm, although the fertilization rate using Atlantic croaker sperm thawed at 0 °C was significantly lower (Gwo et al. 1991). A similar observation has also been made for Pacific herring sperm. A higher thawing temperature (26 °C) was superior to lower temperatures (4, 10 or 18 °C) for thawing of Pacific herring sperm (Pillai et al. 1994). Post-thaw motility of turbot sperm was not different at 20, 30 and 40 °C (Dreanno et al. 1997). In Atlantic halibut sperm, fertilization rates with a low (10 °C) and high temperature (40 °C) were not significantly different when 0.5-mL plastic straws were used (Bolla et al. 1987). For ocean pout, however, there was better post-thaw motility when 1 °C rather than 30 °C was used (Yao et al. 1995). Microwave thawing was used for straws containing black grouper sperm (Chao et al. 1992). There were no significant differences in post-thaw motility of sperm of red drum *Sciaenops ocellatus* when thawed at 10, 20, 30, 40, 50 or 60 °C in a water bath (Wayman et al. 1998).

Sperm Quality

Individual variation in response to cryopreservation of the milt from different males has often been observed (Stoss 1983, Billard et al. 1993, Dreanno et al. 1997). In order to avoid individual effects, sperm from several males were pooled prior to freezing. Explanations other than inherited differences could be responsible for these results because decreases in resistance to cryopreservation are frequently observed towards the end of the reproductive season. The viability during short-term storage of sperm from sea bass (Billard et al. 1977) and Atlantic halibut (Methven and Crim 1991) was less when sperm were collected at the end of spermiation. In turbot, the presence of females enhanced sperm motility during natural spawning. Stripping frequency had no

effect on total volume of turbot sperm, mean sperm motility or sperm number (Suquet et al. 1992a, 1992b).

Egg Quality

A significant decrease (from 53% to 0%) in the fertility rate of Atlantic croaker eggs occurred 2 hr after ovulation (Gwo et al. 1991, 1993b). Therefore, prediction of the time of ovulation is important to the success of artificial insemination due to the short period of viability of the eggs in this species. The eggs of striped bass *Morone saxatilis* must be stripped within 1 hr after ovulation (Stevens 1966) and the eggs of sheepshead *Archosargus probatocephalus* must be stripped within 2 hr (Tucker and Barbera 1987). These reports suggest that the ovulated eggs become overripe if retained in the ovarian lumen, and that timing for stripping of females to gain high quality eggs is species-specific.

Sperm-to-egg Ratio

Owing to the high concentration of sperm and small sperm volumes, fertilizing large numbers of eggs is often difficult with undiluted sperm. Sperm dilution has been investigated as a means to increase the number of eggs that can be fertilized from a small volume of sperm and fertilization rates have generally been higher when dilution was used rather than the dry method (Billard 1985). Fertilization rates appear to be significantly influenced by sperm concentration. Dilution of Atlantic croaker sperm increased fertility at dilutions of 1:5 to 1:50 prior to fertilization, dilution of 1:100 appeared to decrease the fertility (Gwo et al. 1991). The highest sperm concentration gave the lowest fertilization rate of those evaluated, which suggests that high concentrations of sperm lead to unequal distribution of the sperm among the eggs at the time of fertilization (Erdahl et al. 1984). Another theory is that perhaps too many sperm enter the micropylar canal simultaneously or there is an agglutination of sperm in front of the micropyle, thus preventing sperm from entering the egg (Rosenthal et al. 1988). This lack of fertility at higher dilutions may also be related to a scarcity of some essential protein in the sperm extender (Billard 1985) or a disruption of the ionic balance with increased volume of medium (Stoss 1983). Addition of bovine serum albumin to the extender appears to prolong the duration of motility and progressive movement of turbot sperm at high dilution ratios (Suquet et al. 1994). After cryopreservation, the majority of sperm have damaged membranes and mitochondria (Gwo and Arnold 1992, Labbe et al. 1997, Ogier de Baulny et al. 1997). Immediate use of the thawed sperm is often employed for high fertility. One gram of eggs was the maximum which could be fertilized effectively with 0.25 mL of thawed Atlantic croaker sperm (10 parts of 1% sodium chloride to 1 part of sperm) (Gwo et al. 1991). Ten times more cryopreserved sperm than fresh sperm were needed for fertilization in turbot and Pacific herring (Pillai et al. 1994, Dreanno et al. 1997). These results imply that the fertilization capacity of cryopreserved sperm is lower than that of fresh sperm, and that increasing the number of thawed sperm appears to compensate for the decrease in motility. The standard artificial insemination method in sea bass and gilthead seabream were 10 mL of diluent plus 2,000 eggs plus 10 mL sperm (Billard 1978b). Similar practices have been reported for turbot (Suquet et al. 1995) and barramundi (Palmer et al. 1993).

The Effect of Cryopreservation on the Motility of Sperm

When Atlantic croaker sperm (either before freezing or after thawing) were diluted in 3% NaCl at 25 °C, many were instantly motile although swimming stopped within 60 sec (Gwo 1995). There were significant linear relationships between specific motion characteristics and

time after the initiation of motility (Figure 1) (Gwo 1989).

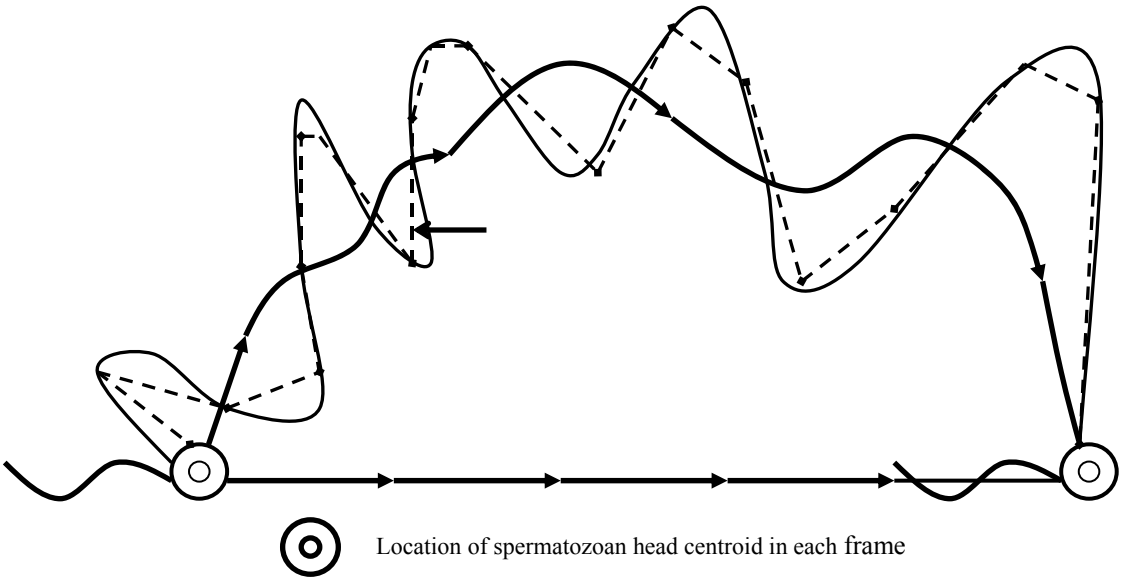

Figure 1. Path of the centroid of an Atlantic croaker spermatozoan head and the parameters measured by the Hamilton -Thorne motility analyzer. Definitions are based on descriptions provided in the user's manual.

Cryopreservation evidently induces alterations in teleost sperm motion. Usually, thawed samples have a lower percentage of motile sperm (Gwo 1989, Dreanno et al. 1997) less head movement and slow circular movements (Gwo 1989). Parameters of Atlantic croaker sperm motion that differed significantly between before freezing and after thawing were percentage of motile sperm, mean progressive velocity, mean path velocity, mean track speed and mean lateral head displacement (Table 2) (Gwo 1989). However, the velocity of fresh turbot sperm did not differ significantly from that of thawed sperm (Dreanno et al. 1997).

Table 2. Motion characteristics* of Atlantic croaker sperm before freezing and after thawing.

Motion Characteristics	Before freezing	After thawing
Percentage of motile sperm**	90.2 ± 4.9[a]	63.1 ± 6.1[b]
Progressive velocity (μm per sec)	81.2 ± 12.2[a]	42.2 ± 13.2[b]
Path velocity (μm per sec)	95.3 ± 15.9[a]	47.3 ± 15.4[b]
Linear index (%)	78.2 ± 7.7[a]	87.7 ± 3.4[b]
Track Speed (μm per sec)	101.9 ± 13.2[a]	55.1 ± 14.3[b]
Linearity (%)	71.9 ± 7.4[a]	78.8 ± 7.5[b]
Lateral head displacement (μm)	3.5 ± 0.4[a]	1.4 ± 0.3[b]

* Values are mean ± standard deviation; N = 6 in all experiments. Different superscripts in a row indicates a significant difference ($P < 0.01$)

**Standardized values at 20 sec after the activation of spermatozoa.

The significant correlation between mean progressive velocity, mean path velocity, mean track speed and mean lateral head displacement were evidence that rapid motion was associated with greater lateral motion of sperm heads (Table 3). Also, there were high coefficients of correlation between the mean progressive velocity, mean path velocity, mean track speed, mean lateral head displacement and the mean percentage of motile sperm. Of the characteristics of sperm motion evaluated, coefficients of correlation with fertilization rate were all highly significant, except mean linear index and mean linearity. The fertilization rate was positively correlated with the mean percentage of motile sperm, mean progressive velocity, mean path velocity, mean track speed, mean lateral head displacement and mean linearity; whereas fertilization rate was negatively correlated with the mean linear index. The mean percent of motile sperm was a better indication of the fertilization rate than any other motion characteristic (Gwo 1989). A regression analysis of the data showed that the best single test was mean percentage of motile sperm ($R^2 = 0.546$).

A fertilization rate model which included mean progressive velocity, mean path velocity, mean linear index and mean lateral head displacement yielded a R2 value of 0.8936. This suggests that, for Atlantic croaker, the number of progressively motile sperm can be the major consideration in developing criteria for predicting the fertilizing ability of sperm.

Conclusions

Visual estimation of sperm motility has not proven to be a good indicator of fertilizing ability. The visual estimate of motility is difficult to express in absolute and objective units of measurement, and is imprecise. Although no post-thaw motility was observed when Atlantic croaker sperm were diluted with 1% sodium chloride extender containing 30% of DMSO, fertilization was obtained with these sperm (Gwo et al. 1991). Immotile thawed sperm of striped bass spermatozoa gave an 88% fertilization rate (Kerby 1983). On the other hand, thawed sperm of common carp and cobia *Racycentron canadum* were motile, but did not fertilize eggs (Kossman 1973, Stein and Bayrle 1978, Billard 1984, Caylor et al. 1994). Although some authors have reported a positive correlation between fish sperm motility and fertility (Mounib et al. 1968, Harvey et al. 1982), most have found little or no relationship between them (Truscott and Idler 1969, Kossman 1973, Stein and Bayrle 1978, Kerby 1983, Gwo et al. 1991). Despite the imperfections of the techniques, there is a general opinion that poor motility is a sign of decreased fertility and motility remains the principal laboratory measurement currently available for evaluating fish sperm quality. Low correlations reported between fish sperm motility and fertility may have resulted from: 1) low accuracy and precision of the visual method for determining sperm motility; 2) imprecision in measuring fertility; 3) influence of factors unrelated to sperm, such as egg quality, and hatchery management, and 4) the influence of other factors on fertility such as damage to sperm that is not detectable by evaluation of sperm motility. Fertilization should be considered as the most effective method to evaluate the quality of thawed sperm.

Table 3. Correlations among various motion characteristics of Atlantic croaker sperm and fertilization.

Motion characteristic	SM2	SM3	SM4	SM5	SM6	SM7	Fertilization rate
Percentage of motile spermatozoa (SM1)	0.878**	0.836**	-0.394	0.879**	-0.212	0.969**	0.739**
Progressive velocity (SM2)		0.989**	-0.576*	0.984**	-0.318	0.956**	0.637*
Path velocity (SM3)			-0.685*	0.987**	-0.437	0.932**	0.578*
Linear velocity (SM4)				-0.638*	0.926**	-0.52	-0.035
Track velocity (SM5)					-0.390	0.954**	0.648
Linearity (SM6)						-0.309	0.137
Lateral head displacement (SM7)							0.702

***Significant at $P < 0.05$.**
**** Fertilization rates were based on late embryos near hatching.**

Future Developments

Viability Assays

A major concern with regard to cryopreserved sperm is the quality compared to that of fresh sperm. Methods for evaluating fish sperm quality and damage during cryopreservation are limited. Many researchers are trying to develop and perfect reliable assays for sperm quality. These methods need to be carefully standardized and each method must ultimately be evaluated by fertility testing. Single laboratory tests of seminal quality are of limited value in predicting fertility because no single physical or biochemical measurement can encompass all of the sperm characteristics necessary for successful fertilization.

Other assays are possibly more sensitive to different types of damage that are of importance to fertility. For example, measurement of ATP synthesis evaluates the integrity of the mitochondria in the midpiece. Lactate dehydrogenase has been identified as a midpiece-associated enzyme which, when released to the extracellular media, can indicate membrane, midpiece and tail damage. Percentages of visually motile sperm and sperm velocity calculations by computerized analysis evaluate the status of the contractile elements of the tail. The loss of motility can be considered to imply a loss of cellular function, but maintenance of motility, however, does not necessary indicate cellular integrity, because some damage to sperm is not detectable by evaluation of motility. Until we can improve laboratory techniques for measuring cell damage, little progress is going to be made regarding relationships of quality and fertility. Because even the best assay methods available today only distinguish large differences, the use of more than one assay to evaluate the status of different areas of sperm for quality analysis is recommended.

Sperm Motility Analysis

In general, fish sperm show two phases of movement: progressive and oscillatory. The latter is observed when sperm move "in place" (stationary vibrations or slow circular motions). Classically, motility of fish sperm in diluted suspensions has been examined with microscopic inspection in a number of fields of view. Typically, researchers examine the intensity of sperm movement, the duration at a given intensity (particularly the initial phase) and the percentage of motile sperm. Many researchers have used an arbitrary scale ranging from 0 to 5 for intensity (Billard 1978a, Doi et al. 1982, Hara et al. 1982, Withler and Lim 1982, Leung 1987). Due to the huge number of sperm per unit volume, irregular levels of dilution, short duration of motility after activation, rapid sperm movement and human error, the accuracy and reproducibility of the results depend largely on the skill and judgment of the observer. The lack of standards for interpretation has contributed to poor repeatability and variation among observers. The availability of a standard, rapid, accurate, reliable and objective method to provide proper interpretation is thus needed.

Several sophisticated systems have been developed for motion analysis of teleost spermatozoa (Billard et al. 1993, Toth et al. 1995, Ravinder et al. 1997). A computerized system to study the beat frequency of the flagellum and head movement tracks has been recently been made available and validated (Ravinder et al. 1997).

Biochemical Changes

Biochemical changes are known to occur during cryopreservation. Seminal plasma biochemical assays may be applicable to fish sperm for evaluation of extenders, sperm

treatments, and freezing techniques when eggs are not available. This would allow elimination of the least effective treatments prior to fertility trials. Additionally, the information on sperm biochemistry gathered in the search for markers indicative of cell damage could be useful in the formulation of sperm extenders.

Bioluminescence techniques have provided evidence for the loss of ATP from the midpiece region of rainbow trout spermatozoa (Ogier den Baulny et al. 1997). Measurements of spermatozoa-associated enzymes and high energy phosphates also have been used to evaluate damage during freezing. Significant increases of 14 amino acids in the seminal plasma of rainbow trout were found after thawing (Schmehl et al. 1987). Significant increases in lactate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase and ATPase were found in rainbow trout sperm following cryopreservation (Lahnsteiner et al. 1996). Inhibition of ATP synthesis via glycolysis and triglyceride catabolism was also found. Lipids were lost from rainbow trout spermatozoa during freezing, and the membrane phospholipid liposomes and the particulate nature of the material released from rainbow trout spermatozoa suggested membrane fragmentation (Labbe et al. 1997). Freezing also appeared to induce profound cation disturbances in addition to causing release of intracellular enzymes. Intracellular levels of sodium and calcium increased in thawed rainbow trout spermatozoa, while levels of phosphorus, potassium and magnesium decreased (Schmehl et al. 1987). Alternatively, calcium and zinc levels decreased in thawed rainbow trout seminal plasma, whereas phosphorus levels increased.

Morphological Evaluation

Morphological studies have revealed that the plasma membrane, mitochondria and flagella of fish sperm can be damaged by cryopreservation. Thawed Atlantic croaker sperm (frozen to -196 °C) had damaged plasma membranes, although the nuclei were left intact (Gwo and Arnold 1992). Moreover, the mitochondria were often damaged or broken and material underlying this region was disorganized. Ultrastructural changes in rapidly frozen rainbow trout sperm occurred in about 20 to 40% of the sperm and showed complete destruction of the cytoplasmic membrane surrounding the midpiece region, coiled tails and swollen mitochondria (Lahnsteiner et al. 1996). No attempt has been made to correlate frequency of damaged spermatozoa to fertilization rate.

Production Requirements

Fish sperm is commonly frozen in 0.5-mL plastic straws or as pellets (0.1 mL). Because cryopreserved sperm has a lower fertilization capacity than does fresh sperm, large volumes of sperm are required. Methods for handling and storage of large volumes of fish sperm are needed. Although many researchers have reported hatching of eggs fertilized with thawed sperm, few experiments have been conducted to show that fish produced with cryopreserved sperm had survival and growth rates comparable with those of fish produced with fresh sperm (Kurokura et al. 1986, Palmer et al.1993, Tiersch et al. 1994). Such studies will be important in the future.

Biochemical Analysis of Seminal Plasma and Sperm

Knowledge of the composition of the seminal plasma and sperm is necessary for a better understanding of the physiology of sperm and improvement in gamete management. When the sperm of certain mammalian species (especially ungulates) are cooled rapidly for a few min, a large proportion becomes irreversibly immotile and infertile. This phenomenon is known as cold shock (Quinn 1985). However, there have been no reports suggesting that fish sperm are

sensitive to cold shock, and fish sperm are usually stored on ice prior to processing. The susceptibility of mammalian sperm to cold shock varies with species. Cold-shock sensitivity of mammalian sperm has been confirmed to correlate with the ratio of polyunsaturated to saturated phospholipids and also to the proportion of cholesterol in the sperm membranes, and is also associated with phase changes affecting membrane function (Anchordoguy et al. 1987, Parks and Graham 1992). It has been suggested that cold- shock susceptibility is determined by membrane cholesterol content and the proportion of polyunsaturated fatty acids that influence lipid fluidity. The lack of susceptibility of sperm to cold shock suggests that the membrane composition of fish sperm is different from that of mammalian sperm. However, little information on the membrane lipids of fish sperm is available. The biochemistry of sperm and seminal plasma has been not established in marine fishes.

Standardization of Protocols

The optimal ratio of sperm and eggs is critical in evaluating the fertilization capacity of fresh and thawed sperm. Because sperm concentration can vary within and among individuals, among species and during breeding seasons, the final concentration of diluted sperm, numbers of sperm and eggs and sperm-to-egg ratios during insemination trails should be standardized. Freezing and thawing rates can have profound effects on the success of cryopreservation. These data are especially difficult to compare among studies because often only the duration of freezing and the temperature used for thawing are reported. The freezing rates generated using liquid nitrogen vapor can be highly variable and are influenced by the size of the straws or cryovials and the position of the sample in the freezing container. Accordingly, a high level of control is desirable for establishing optimal freezing and thawing rates for sperm of different fish species.

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Annotated Bibliography of Developments in the Last Decade

After the publication of this chapter in 2000, additional papers have been published to address sperm cryopreservation in marine fish. Primarily, the species involved were aquacultural species such as trout and sea bream (Cabrita et al. 2005), turbot (Chen et al. 2004, Chereguini et al. 2003), Atlantic cod (DeGraaf and Berlinsky 2004), sea perch (Jia et al. 2004), wolffish (Le Francois et al. 2008), Japanese bitterling (Ohta et al. 2001), flounder (Rideout et al. 2003, Zhang et al. 2003), and ocean pout (Yao et al. 2000), and the research topics mainly address development of sperm cryopreservation protocols. In addition, two reviews have been published since 2000. One addressed sperm cryopreservation in marine fishes in general (Suquet et al. 2000), and the other addressed measurement of factors affecting sperm quality (Rurangwa et al. 2004). These reviews are informative including a summary of a large number of updated publications.

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V. Cryopreservation of Eggs and Embryos of Fishes

Problems and Prospects in Cryopreservation of Fish Embryos

Mary Hagedorn and Fritz W. Kleinhans

Yolk-laden embryos, including those from fishes, reptiles, birds, and amphibians, represent a class of complex, multicompartmental biological systems that have not been successfully cryopreserved. Over the past 50 yr, the fish embryo has presented a challenge to cryobiologists because of its interesting and complex physiology. Despite the lack of success in cryopreserving fish embryos, scientific efforts move forward because of the potential benefits of success to conservation of aquatic ecosystems and to humanity through increased food production. In this chapter, we will explore the complexity of the physiology (which has contributed to the intractability of fish embryos to cryopreservation) and the benefits of successful cryopreservation to aquaculture and conservation.

What Benefits will Cryopreservation of Fish Embryos Bring to Aquaculture and Conservation?

Developing techniques for the cryopreservation of teleost germplasm is timely and the need is pressing. At present, aquaculture is largely dependent upon wild fish populations or continuous maintenance of living cultures. Unfortunately, the availability and productivity of these systems is continually threatened by accidents, natural disasters, breeding failure and disease. For example, valuable genetic lines created for productivity and disease resistance, such as transgenic fish and hybrids (Houdebine and Chourrout 1991), must be maintained in live-culture systems. These strains can take yr to generate, which is costly in terms of space, maintenance, and research effort and are subject to loss through genetic drift. Systematic germplasm cryopreservation can have a profound impact on aquaculture by: 1) allowing the maintenance of large gene pools and reducing inbreeding; 2) reducing pressure on wild populations from collection activities; 3) maintaining a constant supply of animals (i.e. some animals are unavailable in the wild during certain times of the yr); 4) decreasing aquaculture costs by reducing the facilities needed; 5) reducing the impact (e.g. contamination with antibiotics) of aquaculture sites upon wild populations and food resources, and 6) sustaining productivity by minimizing the impact of live-culture failures resulting from human error, natural disasters, breeding failure and epidemics.

In addition to the important scientific and direct applications to aquaculture, cryopreservation of teleost embryos would have considerable conservation benefit to endangered species. Of the estimated 24,600 fish species (Nelson 1994), the greatest number and diversity of fish species inhabit the tropical regions (Wilson 1988). Rapid destruction of tropical ecosystems is a global event and within the next 25 to 50 yr, massive extinctions of endemic species within these areas are predicted (Janzen 1988, Raven 1988). Many freshwater communities in developing areas, including Lake Victoria, the rivers of Madagascar, and the Atlantic rivers of Brazil are in critical condition from human encroachment, causing habitat degradation with many endemic species facing and experiencing extinction. Although not as rich in diversity, aquatic ecosystems in developed areas have similar problems. In North America and Europe, salmon stocks are threatened by competition with hatchery stocks, habitat degradation from deforestation, air pollution-induced acidification, and exclusion from home-stream breeding

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habitats by locks and dams. The development of frozen or 'insurance' populations would preserve genetic diversity and assist efforts to prevent the extinction of wild fish species in natural aquatic ecosystems (Ballou 1992, Wildt 1992, Wildt et al. 1993). Most importantly, these frozen populations would allow the time to reform natural resource policies so that endangered habitats are saved. Then, the embryos could be thawed, cultured and released into the rehabilitated environments.

Why Save Fish Embryos, Instead of Just Sperm?

In general, cryopreservation of fish spermatozoa is successful and practiced regularly in field and laboratory settings (Harvey et al. 1982, Stoss 1983, Leung and Jamieson 1991, Rana 1995). Although freezing of teleost spermatozoa is commonly practiced, the successful cryopreservation of teleost eggs or embryos has not been achieved (Rall 1992). If we can freeze fish sperm easily, why freeze embryos? Cryopreserving spermatozoa, of course, saves only the paternal genome that is not necessarily sufficient to preserve genetic diversity. Molecular studies reveal that both the paternal and maternal genomes play important roles in embryogenesis. At various times during development, methylation of certain genes appears to occur preferentially on the paternal or maternal chromosomes; a phenomenon termed 'parental imprinting.' Methylation correlates with the functional expression of the genes (Reik et al. 1987, Swain et al. 1987). Further, genetic factors that are inherited maternally by the oocyte cytoplasm, such as mitochondrial DNA, also may influence development (Pelegri et al. 1996). Specific, non-Mendelian inheritance via the mother suggests that maternally-derived germplasm must be included in the germ banking process, because these maternal genes are a critical component of genetic diversity. Some genetic manipulations, such as androgenesis (all-paternal inheritance) and chimera production, are possible in teleosts and might protect genetic diversity, but these techniques are extremely labor-intensive and result in low survival rates (Thorgaard and Cloud 1993). The ability to preserve both parental lines through the diploid embryonic genome would greatly improve opportunities for conserving and protecting valuable fish species and argues strongly for cryopreservation of embryos. In principle, the problem could be solved by cryopreserving both sperm and mature eggs, however, mature eggs share many of the same cryopreservation problems as embryos.

What is the Best Way to Resolve the Puzzle of Fish Embryo Cryopreservation?

We contend that a systematic approach, using fundamental cryobiological principles, is the key to achieving such a practical goal. Most of the important technological innovations that have advanced the field of germplasm cryopreservation arose from a sound understanding of the mechanisms of cryodamage and cryoprotection (Mazur 1970, Mazur 1984). Successful cryopreservation of germplasm must address several important issues including: 1) the intrinsic biophysical properties of the cells (e.g. membrane permeabilities and osmotic tolerance limits), and 2) determination of the procedural cryopreservation steps, based on the cell's biophysical properties, necessary to minimize cryodamage and maximize survival (Rall 1993).

Several factors are suspected of complicating teleost embryo cryopreservation (Rall 1993) including: 1) a large overall size, resulting in a low surface-to-volume ratio which could retard water and cryoprotectant efflux and influx; 2) large-sized cells, such as the yolk, which could increase the likelihood of membrane disruption by intracellular

ice formation (Mazur 1984); 3) compartments, such as the blastoderm and yolk, with possibly differing permeability properties; 4) a semi-permeable membrane surrounding the embryo, such as the chorion, that may inhibit water and cryoprotectant influx and efflux (Wallace and Selman 1990), and 5) potential susceptibility to chilling injury (Stoss 1983, Zhang and Rawson 1995).

To successfully cryopreserve an embryo, osmotically active water must exit the cells and an appropriate cryoprotectant must enter the cells. The most basic functions of cryoprotectants require that they not be toxic to the embryo, help stabilize the membranes and reduce the formation of lethal intracellular ice crystals. The remainder of this chapter will concentrate primarily on the issues surrounding the removal of cell water and the introduction of cryoprotectants into cells.

What are Some of the Emerging Answers to this Puzzle?

We are using embryos of the zebrafish *Brachydanio rerio* as a model to understand the cryobiological properties of the complex, multicompartmental teleost embryo. We will relate most of the issues in this chapter to the zebrafish model, but will summarize how appropriate this model might be for other fish species. The zebrafish embryo is composed of two distinct cellular compartments: a large yolk and the developing blastoderm (Figure 1a). The major component of the yolk is vitellogenin, a large phospholipid (~400 kDa) (Mommson and Walsh 1988), stored in membrane-bound vesicles within the yolk. At the onset of development, the blastoderm divides and forms a cap of cells on the large yolk. Two of the major structures begin to develop at about the 1,000-cell stage in the zebrafish embryo (Kimmel and Law 1985). As the yolk syncytial layer (YSL) develops, it replaces the thin (~2 µm thick), nonnucleated yolk cytoplasmic layer (Betchaku and Trinkhaus 1978). The YSL begins to envelop the yolk ahead of the blastoderm, and it surrounds the embryo by ~ 50 to 75% epiboly (Solnica-Krezel and Driever 1994). The segmentation period follows and is staged by the number of muscle somites visible in the tail-region from the 1-somite to the 26-somite stages.

Within the past 15 yr, a large body of physiological, developmental and genetic studies on zebrafish embryos has emerged, however, important questions remain that are critical for the formulation of successful cryopreservation protocols. Specifically, little was known about some of the important biophysical properties of the fish embryo. Filling in these gaps is an important first step toward successful cryopreservation.

Chorion

A potential barrier to successful cryopreservation of fish embryos is the outer protective membrane, or chorion (see Figure 1a). This non-cellular layer is composed mostly of glycoproteins (Hamazaki et al. 1985, Begovac and Wallace 1986) and may hinder water and solute movement into and out of the embryo (Harvey and Chamberlain 1982). Fortunately, the chorion is not necessary for proper development in zebrafish, and it can be removed. Recently, a safe and easy method for removing the zebrafish chorion using enzymes was reported (Westerfield 1993) that may be practical for a wide variety of fish species. To reduce the number of potential barriers to water and solute movement into and out of the embryos, dechorionated zebrafish embryos are often used in cryobiological studies.

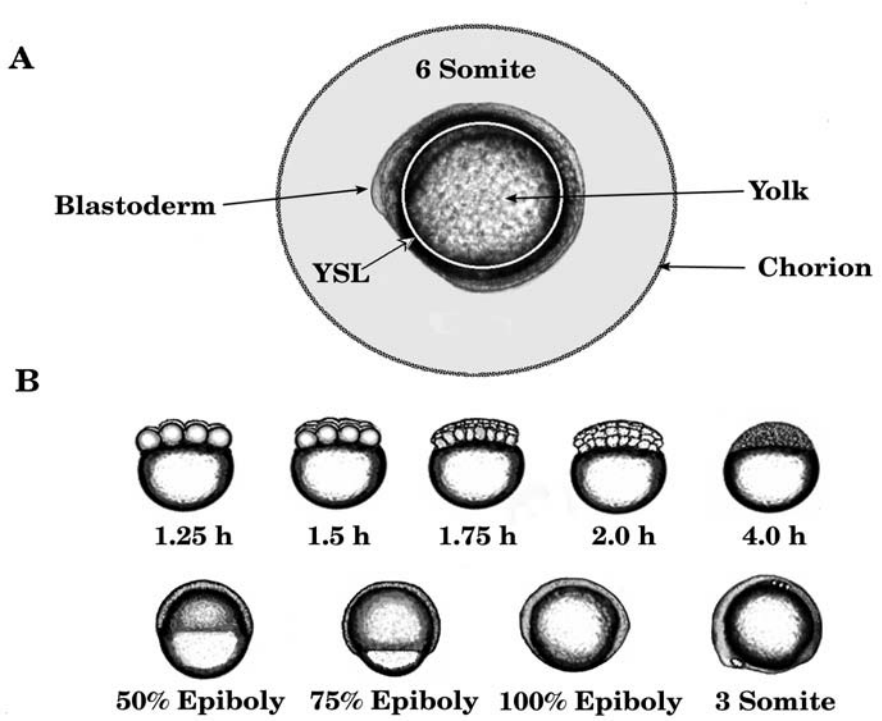

Figure 1: A) Enlarged image of a 6-somite zebrafish embryo identifying the major compartments (yolk and blastoderm). In addition to the main compartments of the embryo, the position of the chorion is indicated although this layer is removed in most of our preparations. Also, the position of the YSL is indicated for clarity, but is not visible at this magnification (from Hagedorn et al. 1997c). B) Drawings depict the developmental stages used in many of these studies (modified from Westerfield 1993).

Chilling Injury or Sensitivity

Cell damage that occurs during cooling but before cell freezing is called chilling injury. The sensitive temperature for each type of cell or embryo varies. For example, zebrafish embryos (at 50% epiboly) held at 2 to 3 °C for 15 min or more fall apart within min after being returned to room temperature (Hagedorn, unpublished data). Although the exact physiological process responsible for chilling sensitivity is not known, it may cause damage to cells in a variety of ways, such as the loss of motility in sperm (Watson 1995), or gross membrane damage, as observed in zebrafish embryos. Stage-dependent chill sensitivity has been reported for embryos of many fish species, including brown trout *Salmo trutta* (Maddock 1974), rainbow trout *Onchorhychus mykiss* (Haga 1982), fathead minnows *Pimephales promelas* (Cloud et al. 1988), common carp *Cyprinus carpio* (Jaoul and Roubard 1982, Roubard et al. 1985, Magnus 1994), zebrafish (Zhang and Rawson 1995, Hagedorn et al. 1997b) and goldfish *Carassius auratus* (Liu et al. 1993). Most of these studies reveal that developmental stages beyond 50% epiboly are less sensitive to chilling injury. We repeated these previous chilling experiments with intact and dechorionated zebrafish embryos, because it was not known if enzymatic removal of the chorion would alter this chilling sensitivity, and we wanted to use dechorionated embryos for future experiments. We found that the enzymatic removal of the chorion did not alter the previously

reported pattern of early-stage sensitivity to chilling in zebrafish embryos (Hagedorn et al. 1997a).

One way to avoid this chilling sensitivity is to 'outrun' the problem by freezing rapidly. When the cells or embryos pass through the sensitive temperature rapidly, cellular damage is reduced. Recent advances in cryobiology by Rall and colleagues have yielded a 'vitrification' preservation method for mice that simplifies embryo freezing techniques while improving the post-thaw survival (Rall et al. 1984, Rall and Fahy 1985, Rall et al. 1987).

Water Permeability

An understanding of the water in the tissues is of primary importance when developing a cryopreservation protocol from first principles. For the zebrafish model, this requires a knowledge, as a function of developmental stage, of the quantity and relative proportion of water and solids in the two main compartments, the fraction of osmotically active water, and the effective permeability of each compartment (the permeability determines how fast water can enter and leave). The stage-dependent compartment volumes were measured with computer-assisted image analysis at various developmental time points (Hagedorn et al. 1997b). To measure water content within this system, traditional cryobiological techniques, such as volumetric measures with light microscopy and wet and dry measurements, and more advanced technology, such as electron spin resonance (ESR) (Hagedorn et al. 1997c) and magnetic resonance (MR) microscopy (Hagedorn et al. 1996) were used. For example, at the 6-somite stage, the yolk compartment comprises 61% of the total embryo volume, and the blastoderm comprises the remaining 39% of the volume (Figure 2). Using the above methods, the yolk compartment contained slightly more solids (58%) than water (42%), whereas the blastoderm was mostly water (82%) and only 18% solids (Hagedorn et al. 1997c).

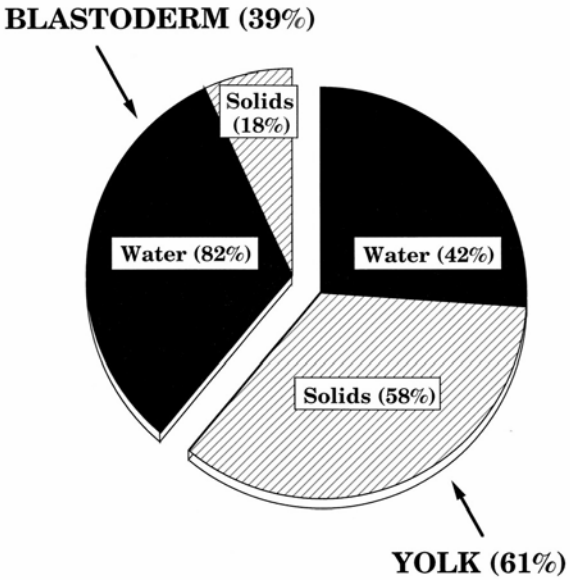

Figure 2: The percentage of water differs in the two compartments of the zebrafish embryo. Also, the distribution of these constituents changes throughout development as the blastoderm grows. In this example, the distribution of the water and solids in the blastoderm and yolk of a 6-somite embryo is shown (from Hagedorn et al. 1997c).

When permeable membranes are exposed to water or solute gradients, a flux of these constituents occurs across the membrane. Until recently, the distribution of water within the blastoderm and yolk, the areas and volumes of these compartments, and their permeability to water (the hydraulic conductivity, L_p) were unknown. The hydraulic conductivity is critical for developing optimal cryopreservation protocols because it controls the length of time required for a cell to dehydrate (Mazur 1970). The water flux across a cell membrane is given by the formula:

$$dV_W /dt = L_pART(M^i - M^e)$$

where V_W is the cell water volume; A, the compartment area (assumed to be constant in this discussion); $L_{P,}$ the water permeability; R, the gas constant; T, the absolute temperature, and M^i and M^e (the intracellular and extracellular solution osmolalities, respectively) (Dick 1966). Reduction of the osmotically active water within the zebrafish embryo is crucial for successful cryopreservation. Dehydration times are dictated by the initial amount of osmotically active water within the tissue and how quickly water can move out of the tissue (water permeability). Because of the compartmental complexity of the zebrafish embryo, it is sometimes necessary to approximate the embryo as a single membrane-bound compartment for modeling and protocol development purposes. In this case, measured permeabilities are referred to as 'phenomenological' permeabilities because they characterize the overall response of the embryo and do not represent the permeability of any specific membrane in the embryo. The phenomenological water permeability of the zebrafish embryo has been determined using optical microscopic measurements of the kinetics of volume change in permeating and non-permeating test solutions (Zhang and Rawson 1996b, Hagedorn et al. 1997c). As noted in the above equation, the cytoplasmic osmolality, M^i, is required to determine L_p. However, an appropriate value for the zebrafish embryo is neither obvious nor easily determined. Usually, M^i equals the osmolality of the growth medium, but this assumption may not hold true for freshwater organisms. Hagedorn et al. (1997c) discuss this issue and some of the perplexing osmoregulatory problems facing many freshwater aquatic organisms without rigid walls.

A conventional technique for assessing water permeation involves light microscopy. These measurements monitor the volume changes caused by the movement of cellular water out of the compartments of the embryo. Using optical microscopy to measure the kinetics of volume changes in hypertonic, non-permeating solutions, we determined the phenomenological water permeability (L_p) in the embryo using a one-compartment model. The internal isosmotic value for the zebrafish embryo is unknown. Therefore, we calculated phenomenological L_p using two alternative assumptions for the internal isosmotic osmolality of the zebrafish embryo: 1) 40 mOsmol, which is equal to the osmolality of the surrounding embryo medium; and 2) 300 mOsmol, which is typical of osmotic values of cells for other vertebrate species (Guyton 1981). These assumptions yield mean L_p valves (± SEM) of 0.022 ± 0.002 µm $min^{-1}atm^{-1}$ for 40 mOsmol and 0.040 ± 0.004 µm $min^{-1}atm^{-1}$ for 300 mOsmol where the non-permeating solute, NaCl, was used (Hagedorn et al. 1997c). These water permeabilities for zebrafish embryos were considerably less than those described for other embryos, such as *Drosophila* (0.722 µm $min^{-1}atm^{-1}$) (Lin et al. 1989) and mice (0.43 µm $min^{-1}atm^{-1}$) (Leibo 1980). Using permeating solutes to measure L_p, Zhang and Rawson (1996 b) found slightly larger values (0.1 and 0.17 µm $min^{-1}atm^{-1}$) for zebrafish embryos in the presence of propylene glycol (PG) and methanol (MeOH), respectively. These values are somewhat larger than Hagedorn et al. (1997c) and, in part, may reflect differences in the modeling parameters used to estimate L_p. Additionally, there is a growing body of evidence that suggests L_p is modified in the presence of permeating cryoprotectants for a variety of membrane types (van Hoek et al. 1990, Gilmore et al. 1995,

Hagedorn et al. 1997c). In zebrafish, Zhang and Rawson (1996b) found a difference of 70% in L_p depending on whether MeOH or PG was used as the osmotic solute. Likewise, we find an order of magnitude reduction in L_p when PG was used in place of NaCl as the osmotic solute (Hagedorn et al. 1997c). In all cases, the reported phenomenological L_p for zebrafish embryos indicate that cellular water moved out slowly. This low water permeability must be considered when formulating dehydration protocols during cryopreservation; otherwise freeze-related damage is inevitable.

Cryoprotectant Permeability

Typically, cryoprotectant concentrations of 1 to 2 M are required for slow-freezing and higher concentrations are required for fast-freezing protocols. However, insufficient information exists on the kinetics of cryoprotectant permeation into the membrane-bound compartments of zebrafish embryos to determine appropriate protocols for loading the embryos with cryoprotectant. One way to measure permeability is to monitor isotope-labeled cryoprotectant permeability over time. Harvey et al. (1983) examined the permeability of zebrafish embryos 5.3 hr after fertilization (50% epiboly) to isotope-labeled dimethyl sulfoxide (DMSO) and found that DMSO permeated into dechorionated and intact embryos. The amount and location (i.e. yolk versus blastoderm) of these permeating cryoprotectants were unclear. Suzuki et al. (1995) reported uptake of isotope-labeled DMSO solution into the perivitelline space and some tissues of fish embryos, but the concentration of the cryoprotectant within the tissues was low and insufficient for successful cryopreservation.

Another conventional technique for assessing cryoprotectant permeation into embryos involves light microscopy. This technique monitors the volume changes caused by the movement of cellular water and cryoprotectants into and out of the cellular compartments of the embryo. Zhang and Rawson (1996a) described the ability of a variety of cryoprotectant combinations to permeate intact 6-somite and 27-hr embryos by measuring volume changes in the outer chorion. They found cryoprotectants passed through the chorion into the perivitelline space, but this method provides no information about the permeability of the yolk or blastoderm compartments. Using dechorionated embryos, Zhang and Rawson (1996b) and Hagedorn et al. (1997b) found volume changes related to permeation, using MeOH as a cryoprotectant at the 70% epiboly and 6-somite stages. Unfortunately, these volume changes were rather small (~ 8%). Often light microscopy experiments fail to measure the small, whole-embryo volume changes that would provide a clearer understanding of cryoprotectant permeability (Hagedorn et al. 1997b). We believe that this reflects the low permeability of the zebrafish embryo to cryoprotectants. However, in some rare cases, a high cryoprotectant permeability yields an influx of cryoprotectant that roughly equals the efflux of water with no detectable volume change. Such a phenomena was observed by Rall et al. (1984), who successfully cryopreserved mouse embryos in 3 M MeOH, but observed no volume change in the embryos over 180 min. Thus, planar, light microscopic measurements must be used with caution, particularly, with complex, multicompartmental structures, such as the zebrafish embryo.

To overcome the problems encountered with light microscopy, MR microscopy was used as a means for assaying cryoprotectant permeability in live zebrafish embryos (Hagedorn et al. 1996). Except for a few special hardware requirements, such as a high magnetic field, strong field gradients, and small radio frequency (*rf*) transmitter-receiver coils (Callaghan 1991), MR microscopy shares the same imaging physics with large-scale, clinical MR imaging instruments.

Although the achievable spatial resolution of MR microscopy (~10 µm) is not comparable to high-resolution light (~0.1 µm) and electron microscopy (~ 1 nm), the MR approach is noninvasive, uses nonionizing radiation, and can be selectively sensitized to specific molecules (Callaghan 1991). Therefore, MR microscopy is particularly well suited for assessing dynamic processes, such as molecular diffusion and permeation in small-sized biological systems. We used chemical-shift selective MR microscopy to visualize the permeation of three commonly used cryoprotectants (DMSO, PG, and MeOH) into 3-somite to 6-somite stage, dechorionated zebrafish embryos (Figure 3a) (Hagedorn et al. 1996). The patterns in the MR images were quantitatively assessed by measuring pixel intensity within and outside the embryo (Figure 3b). These data indicated that the MeOH signal was high initially within the embryo (it takes about

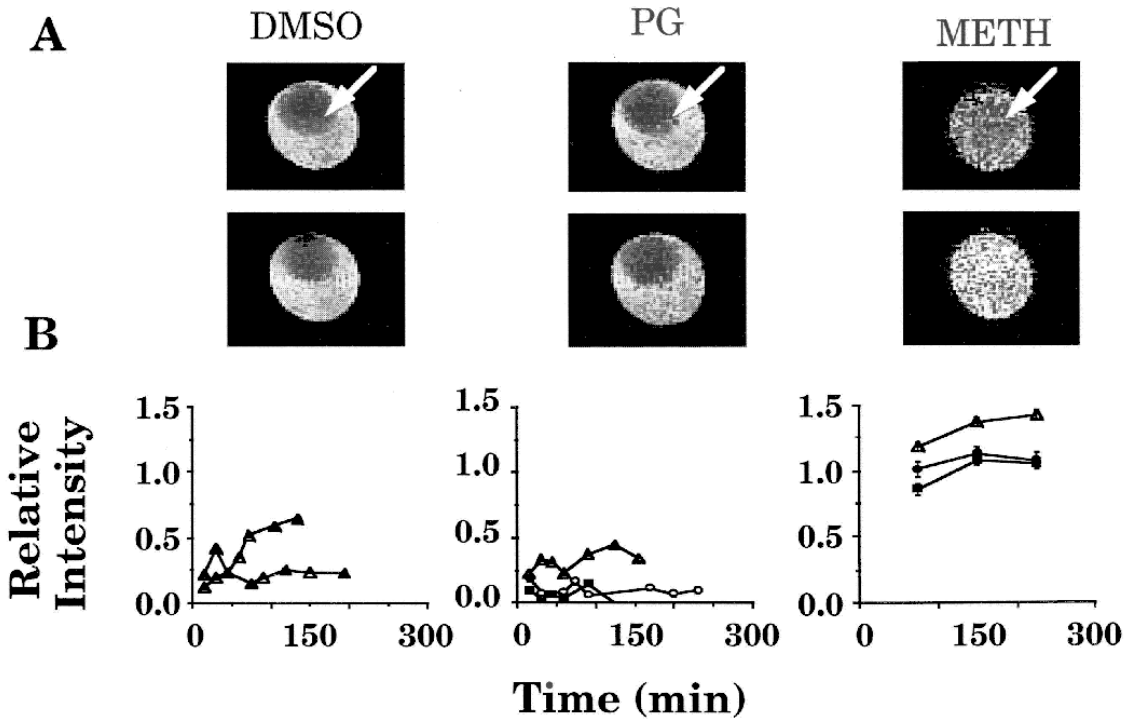

Figure 3: Representative chemical-shift selective MR images of 3-somite dechorionated zebrafish embryos immersed in various cryoprotectant solutions and their relative intraembryonic cryoprotectant signal intensities. The images show a cross-sectional view of the embryo (white arrow) surrounded by the cryoprotectant solution in a glass capillary, all against a dark background void of MR signal. (A-top) Embryos immersed in 2M DMSO, 2M PG, or 2.25 M MeOH in embryo medium for 15 min. (A-bottom) Subsequent images depicting embryos after 2-h exposure to DMSO and PG and 45 min to MeOH. Images of the DMSO- and PG-treated embryos remained dark for 2 h, indicating little or no permeation, whereas images of MeOH-treated embryos showed permeation of large quantities of MeOH. (B) Observations from the MR microimages are quantitatively confirmed by plots of the mean intraembryonic (i.e., yolk) cryoprotectant intensity, normalized to that of the surrounding solution, as a function of post-immersion time. Each symbol represents an individual embryo (from Hagedorn et al. 1996).

15 min to load the embryos and adjust the system). The MeOH signal then increased over time, revealing rapid and continuing cryoprotectant permeation. In contrast, little or no permeation of the DMSO and PG was detected in the embryos.

Potential Barriers to Cryoprotectants

Although MeOH permeates the entire embryo, paradoxically, it may not be as suitable a cryoprotectant as DMSO or PG because it can be toxic at higher concentrations (e.g., 3 to 4 M; Hagedorn, unpublished data). This finding led to an investigation of the physiological mechanisms blocking DMSO and PG permeation into the embryo (Hagedorn et al. 1996). The first question we asked was could the blastoderm block the entry of cryoprotectants into the yolk? To determine if DMSO or PG could permeate the blastoderm, diffusion-weighted MR spectroscopy (the same as MR microscopy in principle but without the imaging field gradients) was used (Hagedorn et al. 1996). These experiments suggested that the blastoderm was permeable to both cryoprotectants (Figure 4).

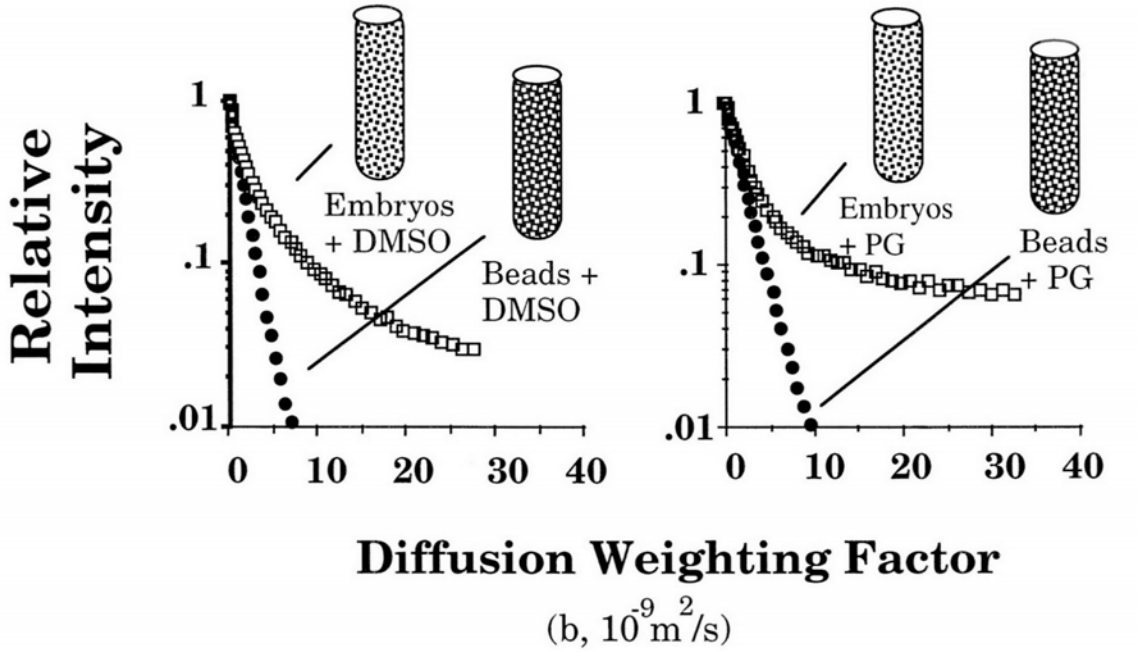

Figure 4: Magnetic resonance signals measured in tubes packed with 3-somite embryos or 160-300 µm diameter nonporous, polystyrene beads immersed in 2M DMSO (left) or PG solution (right). Diffusion-sensitized spectra were obtained, and the cryoprotectant resonance peak normalized to the first point and plotted on a semi-log graph as a function of the diffusion-weighting factor. Cryoprotectant diffusion in nonporous beads exhibited exponential decay (i.e., a straight line on a semi-log plot), characteristic of diffusion in a homogeneous medium. Diffusion of DMSO and PG in embryos demonstrated slower decaying components that became visible at higher diffusion-weighting values. These values deviated from exponential decay suggesting that a significant amount of cryoprotectant had penetrated into the embryo, most likely the blastoderm. This deviation was not a result of restricted diffusion (which may occur in a medium containing closely packed objects) because cryoprotectant diffusion with packed beads had exponential behavior. DMSO and PG exhibited similar diffusion decay curves (modified from Hagedorn et al. 1997a).

The next question we asked was about the contents of the yolk. If the DMSO and PG entered the yolk and bound to some constituent, this would render them invisible to MR. To test the cryoprotectant-binding hypothesis, DMSO and PG solutions were microinjectected into the yolk to determine their detectability and diffusivity within the yolk (Figure 5). Because of the small microinjection volume, the MR signal was weak, and only one-dimensional projection profiles were possible. The profile widths matched the physical width of the yolk, revealing that both cryoprotectants had diffused rapidly throughout the yolk without binding. Furthermore, the signal areas under the profiles (which corresponded to the total amount of cryoprotectant within the yolk) were constant over time. These observations indicated that the cryoprotectants were indeed detectable by MR upon entering the yolk, and they could freely diffuse within the yolk compartment but could not exit (Hagedorn et al. 1996).

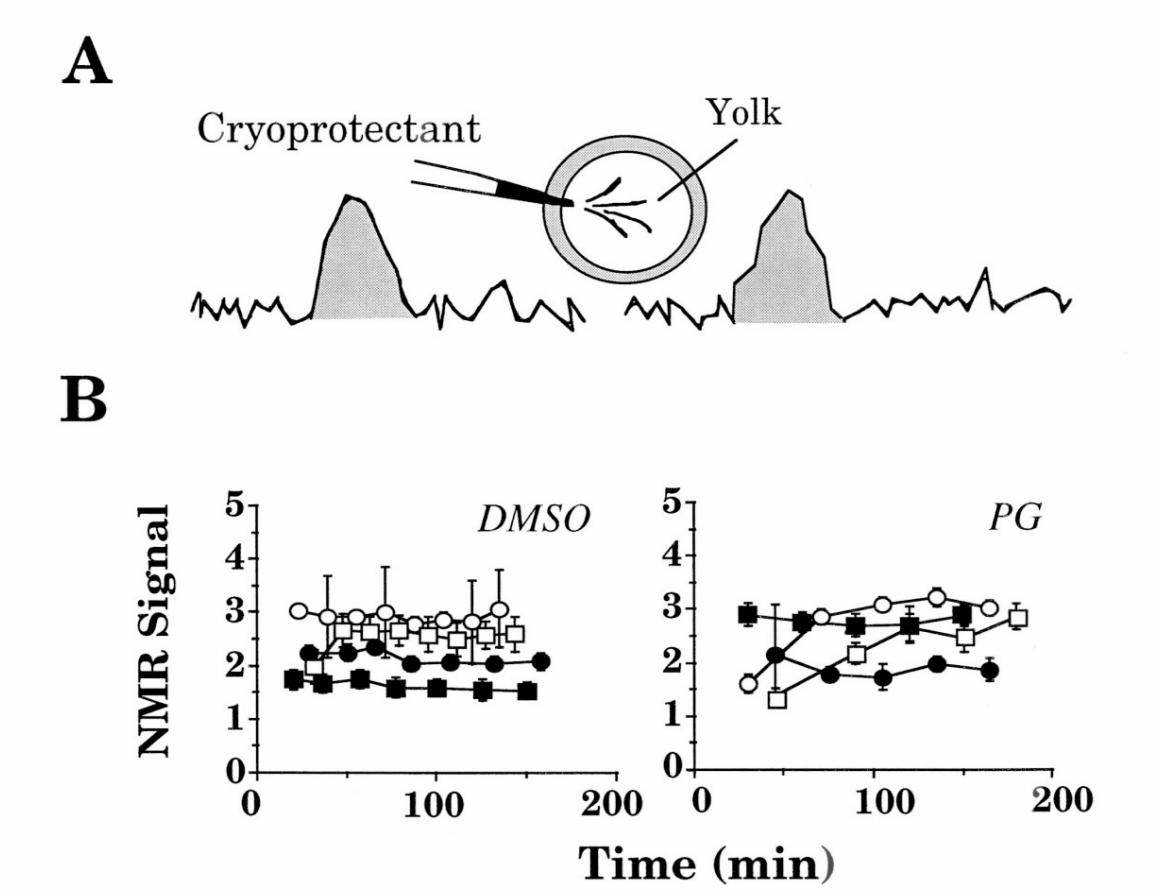

Figure 5: A) One-dimensional intensity profiles of zebrafish embryos with DMSO (left) and PG (right) injected into the yolk. The shaded region of the profiles shows the position of the yolk. Spectra were collected repeatedly across the capillary over a period of 2.5 h to monitor cryoprotectant signal. B) Shaded areas under the intensity profiles were integrated and plotted. The plots show no significant decay of cryoprotectant signal over time, indicating that the cryoprotectant did not diffuse from the embryos. Each symbol represents an individual embryo (from Hagedorn et al. 1997a).

Both these experiments taken together demonstrated that neither the blastoderm nor the yolk contents form a barrier to the permeability of the cryoprotectants. The only other potential barrier is the YSL which develops between the two compartments during epiboly (Figure 1a). Thus these experiments implicate the YSL as the primary barrier to cryoprotectant entry into the yolk. Although many complex factors are involved, we predict that understanding the properties of the YSL will be key to the future successful cryopreservation of zebrafish embryos.

How Applicable is this Model to Other Fish Species?

If we could cryopreserve zebrafish embryos now, how applicable would this model be to other species, especially important aquaculture species? All freshwater and marine teleost fish go through the same developmental events as zebrafish. Therefore, the information learned, especially about membrane permeability to water and cryoprotectants, should be transferable to many other teleost species. In particular, all teleost species have a YSL and go through epiboly. Therefore, we predict that the YSL will act as a major barrier to the movement of most cryoprotectants into the yolk. Most importantly, whatever technical "fix" we devise to overcome this barrier, so that cryoprotectants can enter the yolk in an appreciable quantity, may be applicable to many teleost species, especially the most commonly cultured teleost species in the U.S., such as catfish, salmonids, tilapia, striped bass and sturgeon. Although sturgeons are not teleosts but chondrosteans, they go through epiboly and may well follow the teleost model for cryopreservation (Bolker 1993). On the other hand, these ideas and methodologies may not be as easily applicable to non-teleost species, such as sharks and rays, because they do not go through epiboly.

One criticism of the zebrafish model is that much of the data may not be applicable to important aquaculture species, because some species cannot be removed from their chorion. In considering this criticism, we must ask, what physiological role does the chorion play and how important is it to proper development in most species? Generally, the chorion binds some heavy metals, functions as a filter, and acts as a mechanical barrier to protect the embryo (Depêche and Billard 1994). However, embryos have been raised in culture without chorions and embryos from marine and freshwater species have been successfully dechorionated by mechanical (e.g., *Fundulus spp.* and *Misgurnus fossilis*) or chemical means (e.g., Japanese medaka *Oryzias latipes*, goldfish, northern pike *Esox lucius*, cyprinodontid killifish, and the zebrafish; (Depêche and Billard 1994). This raises the prospect that embryos of many marine and freshwater species may be dechorionated and raised in culture.

At present, a notable exception to the dechorionated culture system is the salmonids. Salmon embryos depend upon the physical support of the chorion during early development, and although chemical and mechanical means are available to dechorionate them, they do not survive this procedure early in development (J. Cloud, personal communication). Only at later developmental stages can the chorion be successfully removed from salmonid embryos. For example, embryos of Atlantic salmon *Salmo salar* at stage 18 (~ 2 wk prior to hatching) can be dechorionated with iridectomy scissors and will continue to grow without developmental problems (Rombough and Garside 1983). For species where the chorion is difficult to remove, we have begun preliminary experiments to puncture the chorion with a chemical dye laser. The chorion remains intact providing support and protection to the organism, yet the small holes allow the movement of water and cryoprotectants into the vitelline space and into the embryo

itself. This may be a faster, albeit a more technical approach, than the one used by Chourrout et al. (1986) who used a micropipette to make small holes in the chorion of rainbow trout eggs.

In the past, it was thought that fish embryos were difficult to cryopreserve because of the impermeability of the yolk itself, rather than the membranes surrounding the yolk compartment. However, as described above, recent NMR studies suggest that the permeability of the yolk may not be an important factor (Hagedorn et al. 1996). These experiments demonstrate that the yolk is freely permeable to cryoprotectants, such as DMSO and PG. Thus species with very large eggs (and correspondingly large yolks), such as the Pacific salmon (Table 1), may have similar yolk-related permeability characteristics as the zebrafish. Clearly, there will be differences and adjustments that will have to be made when applying the zebrafish model to other species. Nevertheless, we believe that the zebrafish model will provide a good conceptual foundation for studies aimed at cryopreserving cultured fish species.

Table 1. Egg sizes of some marine and freshwater teleost species commonly used in fisheries management, basic research and aquaculture. The salmonids are listed as freshwater species because their early developmental stages are in freshwater.

Marine species	**Egg diameter (mm)**	**Reference**
Gadus morhus (cod)	1.1 - 1.9	Blaxter 1988
Pleuronectes platessa (plaice)	1.7 - 2.2	Blaxter 1988
Scomber scombrus (turbot)	0.9 - 1.2	Blaxter 1988
Engraulis mordax (northern anchovy)	1.3 - 1.4	Blaxter 1988
Clupea harengus (Atlantic herring)	0.9 - 1.7	Blaxter 1988
Morone saxatilis (striped bass)*	1.0 - 1.4	Tiersch, pers. comm.
Freshwater species	**Egg diameter (mm)**	**Reference**
Brachydanio rerio (zebrafish)	0.8	Westerfield 1993
Oryzias latipes (medaka)	1.0 - 1.3	Blaxter 1988
Oreochromis mossambicus (tilapia)*	1.7 - 2.2	Blaxter 1988
Ictalurus punctatus (channel catfish)*	4.0	Tucker and Robinson 1990
Acipenser transmontanus (white sturgeon)**	1.0	Bolker 1993
Onchorhynchus mykiss (rainbow trout)*	5.6	Groot and Alerdice 1984
Onchorhynchus nerka (sockeye salmon)*	6.0	Groot and Alerdice 1984
Onchorhynchus gorbuscha (pink salmon)*	6.8	Groot and Alerdice 1984
Onchorhynchus kisutch (coho salmon)*	7.7	Groot and Alerdice 1984
Onchorhynchus keta (chum salmon)*	8.2	Groot and Alerdice 1984
Onchorhynchus tswhawytscha (chinook salmon)*	8.7	Groot and Alerdice 1984

* Important species for aquaculture.

** The sturgeon is a chondrostean and not a teleost, but is an important cultured species and has the same pattern of development as the teleosts (Bolker 1993).

Will Aquaculture Benefit from Cryopreservation of Fish Embryos within the Foreseeable Future?

Although teleost embryos present many challenges for cryopreservation, the studies on water and cryoprotectant permeability presented here, represent a major step toward developing cryopreservation protocols for fish embryos including vitrification. The crucial characteristics of vitrification include: 1) the use of a highly concentrated solution of cryoprotectants, called a vitrification solution, in which embryos are equilibrated and dehydrated prior to cooling, and (2) extremely rapid cooling of the embryo suspension to form a transparent solid or glass. It is noteworthy that *Drosophila* embryos recently have been cryopreserved successfully by

vitrification (Steponkus et al. 1991, Mazur et al. 1992). This achievement has guided our research strategy, because the relative size and complex composition (i.e., blastoderm and yolk compartments) of the *Drosophila* embryo are relatively similar to those of fish (Lin et al. 1989, Rall 1993). The cross-species applicability of this technique has been demonstrated by the successful embryo vitrification of cattle (Massip et al. 1986, 1987), rabbit (Smorag et al. 1989), and sheep (Schiewe et al. 1991). Vitrification also shows great promise as a conservation tool because: 1) it is extremely rapid, so that a large number of germplasm samples can be processed quickly; 2) it does not require sophisticated, computer-controlled equipment, but only LN_2 which is transportable and available in most areas, and 3) most importantly, it may improve post-thaw viability of cryopreserved germplasm (Rall and Fahy 1985, Rall 1992, 1993).

With the proviso that certain permeability barriers can be overcome, notably the barrier to cryoprotectants, this type of technology may well be commonplace within some types of aquaculture within 5 to 10 yr after its success in a model species. In extending success with one model, it will be necessary to test the embryos of each new species for toxic effects and permeability of a variety of cryoprotectants. Not all species respond the same, and testing will take time. Also, successful cryopreservation not only requires that embryos be frozen and thawed successfully, but that they successfully develop and maintain reproductive potential.

Perhaps in the future, local culturists will no longer breed fish, but will purchase frozen embryos from a central distribution point for thawing and maturation in their facilities. We believe that there are many new approaches that will facilitate the understanding and successful cryopreservation of teleost embryos. Once achieved, this could revolutionize aquaculture facilities throughout the world.

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Annotated Bibliography of Developments in the Last Decade

Outside of invertebrates, the status of egg, larval, and embryonic cryopreservation has largely remained unchanged since publication of the first edition of this book as a topic that is unresolved or even considered to be controversial. As indicated in this original chapter, there are strong theoretical constraints to cryopreservation of fish eggs, embryos, and larvae, although publications have reported success, especially in using vitrification (e.g. Tian et al. 2003, Chen at al. 2005, Zhao et al. 2005, Robles et al. 2005, *www.cryogenetics.no*). Often these reports have not involved bringing the samples down to the temperature of liquid nitrogen (-196 ^{o}C), and have not included frozen storage for more than a few hr (e.g., Chen et al. 2005). In addition these findings have not always been reproducible by other laboratories (e.g. Edashige et al. 2006, El-Battawy and Linhart 2009). As such, this remains a problematic area, worthy of much continued study given the tremendous practical benefits offered by cryopreservation of early life stages.

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New Approaches in Cryopreservation of Fish Embryos

Amrit Bart

Overview

The production of many farmed species of fish is dependent on the harvest of broodstock or seedstock from wild populations. Hybrid bass (of the genus *Morone*), for example, is one of the most important cultured fish of the Northeastern United States**,** and their full potential is not realized because of an insufficient seed supply. The continuity of egg and larval supply for farmers concerned with growing fish to table size has been amongst the most important constraints to aquaculture development. A sufficient seed supply available on demand and on a yr-round basis has the potential to revolutionize the aquaculture industry, and to sustain the current rapid growth (Bromage 1995). Successful cryopreservation of commercially important fish embryos using relatively simple methods would provide a means for meeting these needs.

Another additional significant benefit of cryopreservation is the ability to store or "bank" genetic material for extended periods of time at a relatively low cost. Once genetically valuable strains of fish are developed, a minimum number of adult fish must be maintained to prevent genetic deterioration. Maintenance of these stocks is costly, labor intensive and requires space. Furthermore, individual strains may be lost by impaired reproductive efficiency, disease or accident. Cryopreservation would maintain germplasm free of genetic contamination and degradation (Rana 1995). Gamete or embryo cryopreservation has other applications including greater ease in conducting selective breeding for disease resistance and stock improvement, transfer of animals from one hatchery to another, and enhanced propagation where there is a deficiency of males or females. It would also reduce the hatchery operation to only incubation and rearing, making larval production simple and thus, more practical for foodfish producers to operate their own hatcheries.

Recent Advances in Embryo Cryopreservation

Cryopreservation of embryos involves a series of complex and dynamic physiochemical processes of temperature and water transport between an embryo and the surrounding medium. Understanding these processes and their interactions with embryo biology is essential prior to developing a successful cryopreservation methodology. Unfortunately, the technologies developed for mammalian embryo cryopreservation are unlikely to be successful for multi-compartment, highly differentiated and yolk-filled embryos, such as those of fish. Although methods for cryopreservation of fish sperm have been developed for over 80 species of fresh and saltwater fish (Leung and Jaemison 1991, Rana 1995, Figiel and Tiersch 1997, Bart et al. 1998), cryopreservation of eggs or embryos has not been successful and consequently there are few publications on this topic.

The earliest reported cryopreservation of eggs or embryos dates back to 1953 (Blaxter 1953). Zell (1978) and Erdahl and Graham (1987) reported viable eggs and embryos protected with 8 to 14% dimethyl sulfoxide (DMSO); eggs were frozen at -20 °C and embryos at -55 °C. Neither of the reports specified the rate of cooling. It appears that these eggs and embryos were supercooled and not cryopreserved. A more recent set of studies has examined chilling injuries

Bart, A. 2011. New Approaches in Cryopreservation of Fish Embryos. *In: Cryopreservation in Aquatic Species*, 2nd edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 503-512.

(Liu et al. 1993, Zhang and Rawson 1995) and cryoprotectant toxicity at various stages of development (Robertson and Lawrence 1988, Liu et al. 1993, Suzuki et al. 1995). Using magnetic resonance and electron spin resonance microscopy, Hagedorn et al. (1997) found that cryoprotectant permeated into zebrafish embryos. However, the permeation was not uniform due to the yolk synctial layer (YSL) and differentiating blastoderm cells. The permeation rates at the six-somite stage were 42% in yolk, 82% in blastoderm and 74% in whole embryo. While membrane permeability was constant up to 75% epiboly stage, 8 hr after fertilization to the 3-somite stage, the rate of permeation increased two-fold at the 6-somite stage, indicating increased levels of permeation at a later developmental stage.

Stages of Development and Cryoprotectant Toxicity

The ability of an embryo to withstand cryoprotectant toxicity as well as chilling injuries at later stages of development (post-epiboly) is supported by our findings. In our studies at the Center of Marine Biotechnology at the University of Maryland, embryo survival of zebrafish *Brachydanio rerio* was compared at 60% epiboly and at the 8-somite stage. After being immersed in 40% methanol (MeOH), DMSO, ethylene glycol and glycerol (for 5, 10 or 20 min.), embryos treated with 40% MeOH had the highest survival. Longer immersion periods resulted in higher mortality. This indicates that embryos at the epiboly stage of development apparently are more vulnerable to cryoprotectant toxicity than are embryos at the somite stage of development. This also suggests that epiboly to 8-somite stage are better able to tolerate methanol treatment compared with the other cryoprotectants tested (Figure 1).

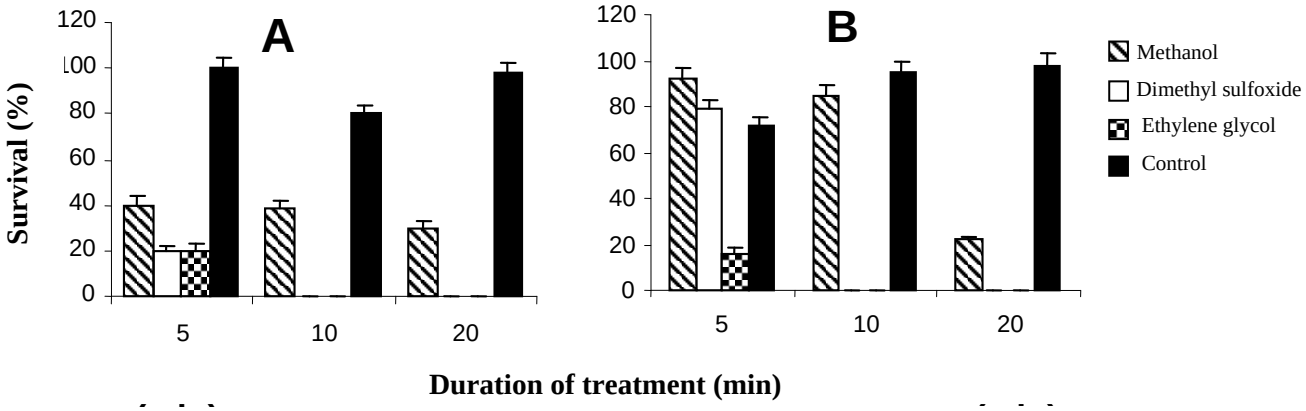

Figure 1. Mean (± SEM) survival of zebrafish *Brachydanio rerio* embryos at 60% epiboly (A) and 8-somite stage (B) treated with three cryoprotectants for 5, 10 or 20 min.

Ultrasound Enhanced Permeation

Successful cryopreservation of embryos requires a clear understanding of the kinetics of cryoprotectants. The permeation of a specified amount of cryoprotective additive (CPA) into each cell within the embryo is critical. Insufficient or uneven permeation can result in localized cell death and subsequent developmental abnormalities. More novel techniques are required to achieve a controlled rate of facilitated diffusion. Cavitation-level ultrasound has shown promise

to increase the transmembrane permeation of relatively large protein molecules (Mitrogotri et al. 1995a).

Cavitation-level, low frequency ultrasound has been used to erode metallic surfaces, to shatter kidney stones, to produce sonoluminescence and to accelerate chemical reactions (Macanshetty 1995). Cavitation occurs in a fluid medium and is induced by low frequency ultrasound (Apfel 1981, Prosperetty 1986). This process involves a sequence of nucleation, bubble formation, oscillation, and the implosive collapse of air bubbles. It results from an ultrasound wave which produces alternating areas of compression and rarefaction, and, consequently, pressure changes in the fluid (Williams 1983). The cavitation effects are observed with the onset of ultrasound at 20 kHz and cease at frequencies greater than 2.5 MHz. Recent studies in human skin tissue demonstrated that these oscillations and the collapse of bubbles effectively disorganize the lipid bilayer allowing larger protein molecules to diffuse through (Mitragotri et al. 1995b). Because more than 70% of a fish embryo is water and the chorion and YSL surrounding the embryo and yolk are in fluid medium ultrasound is expected to enhance permeation across these barriers in a manner similar to that of human skin.

Using techniques similar to those used by Mitragotri et al. (1995b), we used ultrasound (20 to 40 kHz frequency and 30mV amplitude) to determine the rate of diffusion and mortality and the subsequent rate of diffusion (Bart 1998, Bart et al. 1999). An initial series of experiments were conducted in zebrafish embryos to assess the level of toxicity to some of the commonly used cryoprotectants at various concentrations. The cryoprotectants, DMSO, MeOH, ethylene glycol and glycerol at concentrations ranging from 5 to 60% were used to treat four different developmental stages of embryos. Embryos treated with MeOH concentration as high as 60% resulted in nearly 20% viability (Figure 2).

Microscopic examination revealed limited physical damage with this treatment. To assess the rate of permeability with the use of ultrasound we immersed embryos in 300 and 900 mg/L of calcein (2,4-bis-di[carbomethyl]-amino-methyl, MW 622) inside a 2.8-mm diameter cylindrical ceramic piezoelectric crystal transducer. An amplitude of 300 mV at 40 kHz frequency was applied for 10 min in a continuous pulse. The test compound calcein was used as a marker due to its small size, fluorescence, calcium-binding and lack of toxicity properties (Wallach et al. 1959). Treated embryos were dechorionated, homogenized and centrifuged. Extracted calcein in solution was quantified using a multi-plate reader (Cytofluor 4000). Chelation and digestion were achieved with a mixture of 0.01 M EDTA in a standard TBS and 1% Triton X-100. The rate of permeation was found to be significantly different ($P < 0.05$) between the two calcein concentrations, and between those embryos treated with and without ultrasound.

Analyses of calcein content in embryos was performed immediately, 24 hr after treatment and after manual removal of chorion. A several-fold increase in calcein absorption was observed when embryos were treated with ultrasound and with the higher calcein concentration (Figure 3). With this level of ultrasound treatment, mortality was less than 2% and was not different from that of the control group. This clearly indicates that further studies are needed to assess the rate of permeability and localization of cryoprotectants using ultrasound to enhance delivery.

Future Research Studies

Chilling Injury

Chilling injury needs specific attention (Mazur et al. 1992a, 1992b, Zhang et al. 1995).

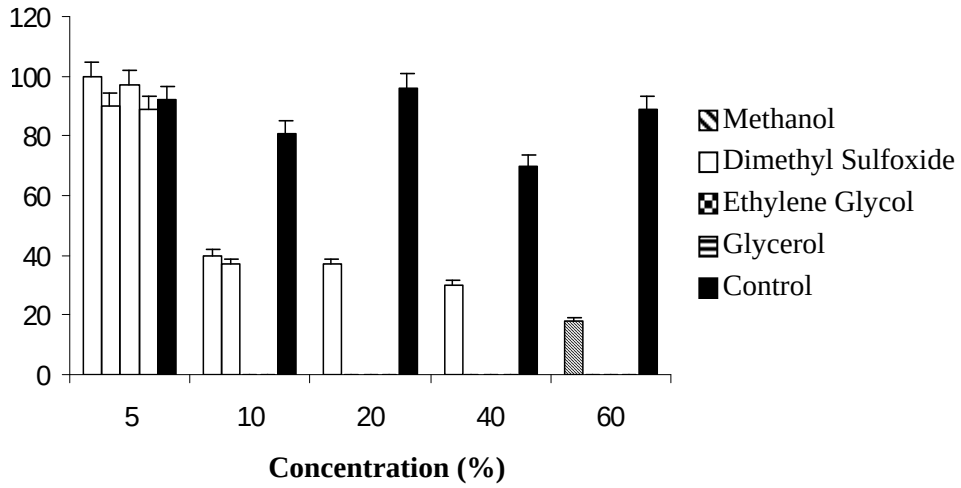

Figure 2. Survival of zebrafish embryos (at 60 to 90% epiboly) after cryoprotectant treatment.

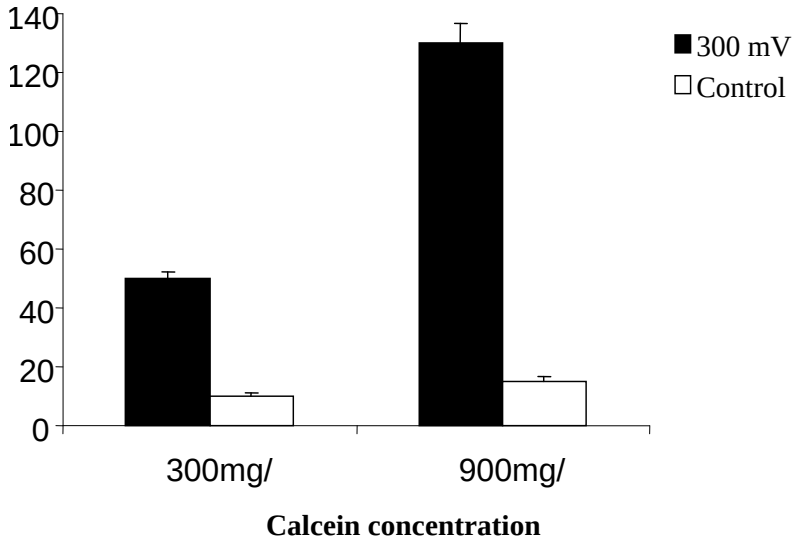

Figure 3. Ultrasound-enhanced diffusion of calcein into zebrafish embryos (Bart 1998).

Sensitivity to chilling injury in some species has varied as a function of embryo stage (Mazur et al. 1992, Zhang and Rawson 1995). New studies should be performed to establish the presence or absence of extracellular ice and to optimize cooling and warming rates as well as holding temperatures. Sensitivity to cold shock requires the use of slow cooling rates, while sensitivity to chilling injury necessitates rapid cooling.

Ultrasound and Electroporation

Further investigation of ultrasound and electroporation enhancement of permeation of CPA is needed to optimize the rate of diffusion across various membrane barriers with minimal damage to the embryo. Ultrasound can increase the rate of permeation (Bart 1998, Bart et al. 1999). Further study is required, however, to examine the localization and the rate of permeation of CPA in the embryo.

Electroporation has previously not received much attention in cryopreservation. Electroporation is an electrical phenomenon known to dramatically and reversibly alter lipid bilayer permeability (Prausnitz et al. 1995). It involves application of a brief electric field pulse (Weaver 1993). Electroporation enhances the permeability of a cell by several orders of magnitude**,** and also enhances transmembrane molecular transport. Although the method has an excellent potential for facilitating transfer of CPA, gene transfer procedures have resulted in high mortality rates. Adjusting various parameters (e.g. amplitude, pulse time, burst time and cycle numbers) could potentially optimize for cryoprotectant penetration.

Soft Chorion

The chorion enclosing the fish embryo is one of the primary barriers to the exchange of solutes. Harvey and Ashwood-Smith (1982) demonstrated that diffusion of isotope-labelled DMSO into the fish embryo increased several-fold when the chorion was manually removed. Masuda et al. (1991) described the mechanism of chorion hardening of medaka eggs. When Ca^{++} ionophore-activation was inhibited by cadaverine, the hardening process was arrested. With the addition of Ca^{++} to the medium, hardening resumed, demonstrating that Ca^{++} is important to the hardening process in the isolated chorion. A number of studies have incubated embryos in calcium-free solutions to inhibit chorion hardening prior to microinjection and electroporation (Yoshizaki et al.1991, Muller et al. 1992, Powers et al. 1992, Knibb et al. 1994). An underdeveloped chorion may permit cryoprotectants to diffuse across the embryo more easily. These calcium ion-deprived embryos have not been subjected to cryoprotectants such as those developed to assess the rate of diffusion alone and in conjunction with other techniques.

Effective CPA Toxicity

Cryoprotectants are toxic to embryos at high concentrations and long exposure times (Shafer 1981). Under these conditions, cellular proteins can be denatured and pre-freezing viability is reduced (Harvey 1983). Even greater damage can be observed when equilibration time is increased. However, it should be noted that this “absolute toxicity” for the CPA is not the relevant criterion for the development of procedures such as vitrification. More important is the effective toxicity of the CPA. Effective toxicity is a function of the permeation rate of the cryoprotectant, the concentration required for the CPA to be effective, the temperature and the duration of exposure. For example, a rapidly permeating CPA with a high absolute toxicity may be preferable to a slowly permeating but less toxic CPA because the former requires a shorter exposure time. Information on the comparative toxic effects of the most commonly used

cryoprotectants on fish eggs and embryos is lacking. In addition, a compound, which is more effective at enhancing vitrification, and thus requires a lower concentration, may be preferable to a CPA with a lower absolute toxicity, but a higher required concentration. A high concentration of a non-permeating, nontoxic CPA can appear to be toxic as a result of osmotic damage to the embryo. Prevention of this confounding factor requires that effective permeabilization protocols be developed prior to toxicity studies.

Vitrification Considerations

Most nontoxic cryoprotectant concentrations fall between 7% and 15% (Rana 1995), an insufficient level for vitrification. Therefore, the embryos need to be loaded with a vitrification solution using a two-stage procedure. First, a low concentration vitrification solution should be loaded into the embryo. This step induces little damage. The embryo can then be placed into the final vitrification solution for periods near to those at which toxicity becomes significant. While the cryoprotectant permeates the embryo slowly, the water permeation is comparatively rapid. The resulting osmotic flux of cell water from the embryo rapidly increases the solute concentration to levels appropriate for vitrification.

If the experimental results indicate problems with the stability of vitrified samples, additional components may be added to the vitrification solution. The stability can be increased by the introduction of higher molecular weight compounds. These compounds are not usually capable of permeating the cell membrane. The addition of polyvinylpyrrolidine and bovine serum albumin, each of which exhibits low toxicity to cells, needs to be examined. These compounds have also been demonstrated to have a substantial vitrification enhancing effect (Mazur et al. 1992, Steponkus et al. 1990). Glucose, skim milk and egg yolk have been shown to be effective in the development of CPA of fish spermatozoa and should be considered for fish embryos (Harvey and Ashwood-Smith 1982, Harvey 1983, Chao et al. 1986).

To maximize the probability of vitrification and minimize the probability of devitrification, the frozen samples should be cooled and warmed as rapidly as possible without inducing thermal stress fractures in the glass or thermal shock in the embryos. Possible techniques for cooling include direct immersion of the storage container in LN_2 or in a mixture of solid and LN_2. Both of these techniques will cool French straws below the glass transition temperature of aqueous glasses (usually around -130 °C). However, the latter technique generates a substantially higher cooling rate.

Conclusions

Clearly, cryopreservation of embryos is significantly more complex and thus more challenging than for spermatozoa. Cryopreservation of embryos presents problems related to permeation and uniform distribution of cryoprotectants at a level not faced while preserving sperm cells. Despite significant progress made in our understanding of teleost embryo biology over the last decade, more information is needed to successfully cryopreserve fish embryos. Fundamental issues remain unresolved. For example, sufficient information on suitable CPA concentrations, rates of diffusion, levels of toxicity, duration of treatments for specific species, chilling injuries, formulation of suitable vitrification solutions, and freezing and thawing rates are lacking in current literature.

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Annotated Bibliography of Developments in the Last Decade

Many new techniques have been employed in the quest to preserve embryos of various fish species since the original publication of this chapter. These techniques include expression of aquaporin channels, vitrification, and microinjection and ultrasound of cryoprotectants. New techniques have also been developed to assess permeability of the chorion and other membranes to water and cryoprotectants.

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Cryopreservation of Sperm and Eggs of African Catfish Formerly Known as Sharptooth Catfish

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The sharptooth catfish *Clarias gariepinus* is an important farmed fish species that has its origins in Africa. The species can produce large amounts of good quality meat, lives in various environments, is highly adaptable, is tolerant of poor water quality and can reach market size in a short time (Hecht et al. 1996). This fish is of great importance in the food supply of Africa and recently has been introduced in Asia (Tripathi 1996). Cryopreservation of sharptooth catfish sperm and eggs has both economical and scientific importance and the results might be adapted to other farmed catfish species such as the North American channel catfish *Ictalurus punctatus*.

Cryopreservation of Sharptooth Catfish Sperm

One of the reasons why effective sperm cryopreservation techniques were needed in sharptooth catfish, among the well-known benefits of long-term conservation, is that this species, like many other catfishes, is oligospermic. The volume of the sperm collected even after hormonal stimulation is generally low. In case of insufficient sperm availability, males are sacrificed, and the testes are removed and cut into small pieces. The milt is extracted by suspending the pieces in a saline solution or by pressing them through a net fabric onto the eggs (Legendre et al. 1996). Sometimes this makes propagation risky, because the amount of sperm obtained may not be sufficient. Cryopreserved sperm would be available if fresh milt was not available.

According to the literature, the cryopreservation of sharptooth catfish sperm is relatively less studied than in other farmed fish species. The first freezing experiments for sharptooth catfish sperm were carried out by Steyn et al. (1985). They froze samples on dry ice and obtained 40% post-thaw motility using 5% glucose as an extender and 5% glycerol as a cryoprotectant (CPA). Sperm stored for 14 d before thawing yielded 51% hatching and sperm stored for 16 mo yielded 41% hatching (Steyn and Van Vuren 1987b). The best results were obtained with a 20-min equilibration in a solution of 5% glucose and 1% or 11% glycerol. Van der Walt et al. (1993) investigated the effect of sperm cryopreservation on the genetic diversity of the species. Steyn (1993) studied the optimal concentrations of CPAs and the most suitable combinations of extenders and CPAs, and found that a combination of 4% glucose and 9% glycerol and a freezing rate of 5 °C per min yielded the best results. Incorporation of an isothermal period into the freezing program led to an increase in sperm survival when dimethyl sulfoxide (DMSO) was present in the freezing solution.

In our experiments we failed to repeat these results with the methods described by Steyn (1993). This might indicate differences between the environmental and nutritional conditions of sharptooth catfish populations farmed in Europe and Africa. The goal of our experiments was to find an efficient, simple, and inexpensive method for cryopreservation of sharptooth catfish sperm suitable to be applied to farm conditions.

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Evaluation of Extender Solutions

We evaluated six extenders: solutions of glucose, fructose, sucrose, NaCl, KCl and their mixtures. The solution containing saccharides and ions was composed to be similar to the seminal plasma of the sharptooth catfish, described by Steyn and Van Vuren (1987a). As a first step, the lowest immobilizing concentration of these solutions was examined. The lowest concentrations which immobilized the sperm in 1:15 dilution were 6% glucose, 6% fructose, 10% sucrose, 1% NaCl and 1.3% KCl.

Artificial seminal plasma (Steyn and Van Vuren 1987a) also immobilized the sperm. Interestingly, the 5% glucose solution described by Steyn (1993) did not immobilize the sperm of our males.

Effect of Buffer Systems

We examined, for the first time, the effect of different buffer systems on the motility and fertilizing capacity of sharptooth catfish sperm. The six extenders were tested with two different buffer systems: Tris-HCl and $NaHCO_3$. The pH of the systems were adjusted to 7.73, the same as that of the seminal plasma of sharptooth catfish (Steyn and Van Vuren 1987a). The optimal concentration of Tris with glucose extender was established experimentally and used in further experiments. The pH value was adjusted with concentrated HCl. In the case of the $NaHCO_3$ system the quantity of $NaHCO_3$ added to the solution established the desired pH value. A total of 18 different extender combinations were tested. The sperm were diluted with the given extender in a 1:1 ratio in 1.5-mL Eppendorf tubes at 0 to 0.2 °C on melting ice for 10 min. Dimethyl sulfoxide was used as the cryoprotectant in these experiments, based on our preliminary observations and literature data. The low temperature of the dilution step was essential to reduce the toxicity of DMSO. The samples were loaded in 0.25-mL plastic straws and cryopreserved in a CRYOCELL-15 (BLS, Hungary) programmable freezer, according to Steyn and Van Vuren (1987b) and Magyary et al. (1996a). Further details of the procedure are described in Urbányi et al. (this volume). After freezing, the sperm were thawed immediately or stored for an additional 2 to 3 d in liquid nitrogen (LN_2).

The post-thaw motility of sperm was evaluated in the 18 extenders and buffer system combinations (Figure 1). The best motility (25%) was obtained with 6% fructose in $NaHCO_3$ buffer solution. Solutions with $NaHCO_3$ buffer had significantly higher motility than did those with or without Tris-buffer. No significant difference was found between the fructose and glucose in the $NaHCO_3$ buffer solutions, although we chose the fructose solution for further experiments.

Cryoprotectants and Equilibration Time

The following step of our experiments was to find the optimal cryoprotectant and equilibration time. Six CPAs were tested using different concentrations and equilibration times: 5, 9 and 11% glycerol; 5, 10 and 15% methanol, ethylene glycol and propylene glycol; 5, 7.5, 10, 12.5 and 15% DMSO and dimethyl acetamide (DMA).

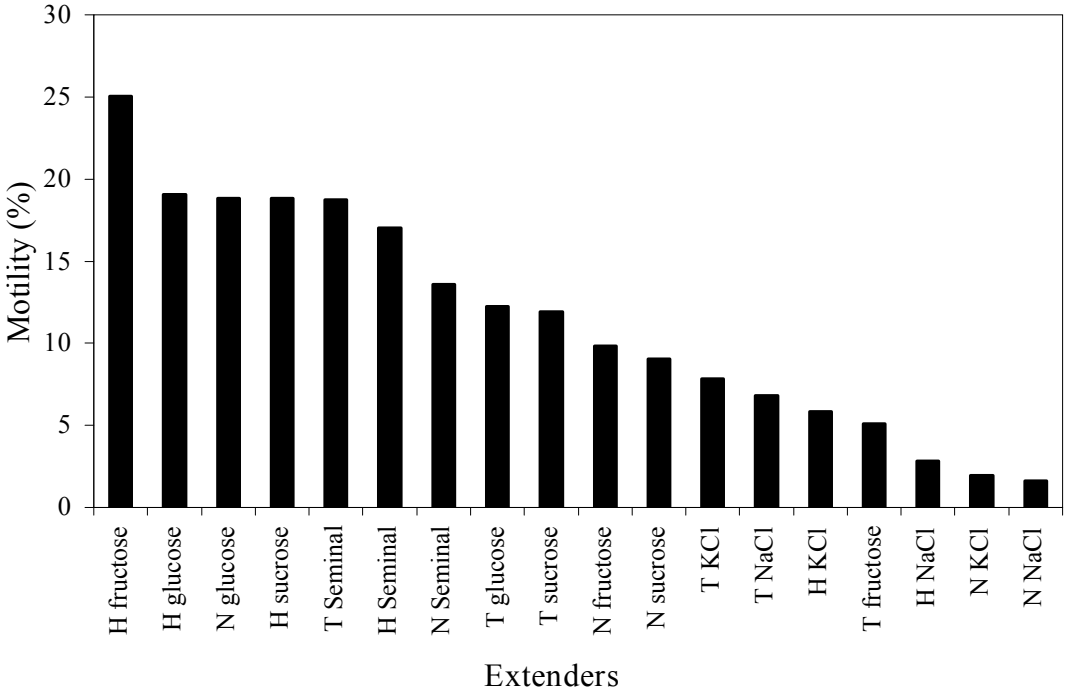

Figure 1. Percent motility of thawed sharptooth catfish sperm diluted with different extender-buffer systems. Abbreviations: H: $NaHCO_3$ buffer; T: Tris-HCl buffer; N: no buffer.

The concentrations of glycerol were chosen according to the literature (Steyn et al. 1985, Steyn and Van Vuren 1987b, Steyn 1993), the other concentrations were an experimental series. A 1:1 dilution ratio of 6% fructose in $NaHCO_3$ buffer solution was used as an extender. The sperm were diluted in 1.5-mL Eppendorf tubes on melting ice. Equilibration time varied between 0 and 40 min. The freezing method was the same as described above. Samples which showed poor motility after equilibration (e.g. ethylene glycol) were excluded from freezing. The best post-thaw motility results were obtained using 10% DMSO (50% motility) or 10% DMA (36% motility) after 10 min equilibration (Urbanyi et al. 1999). Shorter, 5 min equilibrations decreased post-thaw motility by an average of 8 to 10%. Contrary to earlier reports in this species (Steyn et al. 1985, Steyn and Van Vuren 1987b, Steyn 1993), glycerol yielded poor motility.

Fertilization Experiments

Thawed sperm with the extenders and CPAs, yielding the best motility were chosen for fertilization experiments. Different concentrations of sperm were tested (data not shown). One straw of thawed sperm was sufficient to fertilize 1 mL of eggs (~1,000) in Petri dishes. Two fertilization methods were compared: dry fertilization, which is the standard method used in hatcheries and wet fertilization (VI. Some Basic Methods, pp. 264-301). Best results (96% of non-frozen control, 90% of eggs fertilized) were obtained with 10% DMA and wet fertilization, though all fertilization results were above 90% of control, and no statistical differences were observed. Using the most efficient method, fertilization rates with thawed sharptooth catfish sperm would be suitable for farm application. Further experiments are needed to simplify the freezing procedure and eliminate the need for a programmable freezer.

Cryoprotectant Toxicity and Vitrification of eggs of Sharptooth Catfish

Long-term preservation of teleost eggs at the temperature of LN_2 represents an unsolved cryobiological problem. Vitrification might offer a solution to overcome the difficulties that are related to the large size, yolk content, and chilling sensitivity of fish eggs (Rall 1993). Knowledge of permeability to water and CPA, chilling sensitivity of the eggs, and CPA toxicity are essential for design of cryopreservation protocols. The sharptooth catfish is a particularly good species for egg cryopreservation experiments, as large quantities of "dry" unactivated eggs can be obtained without sacrificing the female.

Chilling Sensitivity

Chilling sensitivity, an increased sensitivity to low temperature, has been reported in several teleost species and is considered to be one of the obstacles for cryopreservation of yolk-rich teleost embryos. Our experiments on sharptooth catfish showed that morula-stage embryos were sensitive to exposure to 0 °C and 4 °C for 15 min, however, this sensitivity was not detected in the older, half-epiboly, gastrula, tailbud and heart-beat stages (Figure 2) (Dinnyes et al. 1996). Our further experiments demonstrated that unactivated sharptooth catfish eggs were moderately sensitive to 0 °C (Figure 3) (Baranyai 1995).

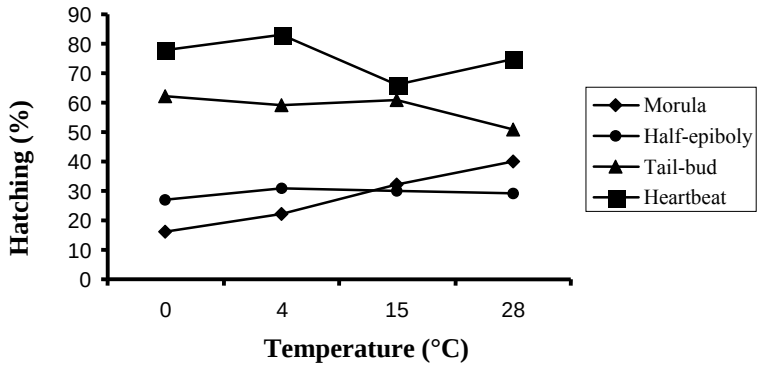

Figure 2. Hatching of sharptooth catfish embryos following a 15-min exposure to low temperatures.

Cryoprotectant Permeability

The mechanisms responsible for the permeability changes in teleost eggs and the means to change those parameters are mostly unknown, providing a good example of the difficulties responsible for the lack of success in fish egg cryopreservation. Fish eggs differ from mammalian embryos in that they are constituted of several very different compartments surrounded by different osmotic barriers (Hagedorn et al., and Bart, this volume). The chorion, the cell membrane, and the syncytial layer, connecting the yolk compartment to the embryonic cells, have very different water and CPA permeability coefficients. Those parameters change during development (Zhang and Rawson 1996a). The resulting multicompartmental system cannot be examined accurately by the traditional method of light microscopic detection of osmotic volume changes due to water and CPA movements. Technically demanding methods for direct measurement of CPA penetration, like radiotracer studies (Harvey and Ashwood-Smith 1982) and magnetic resonance microscopy (Hagedorn et al. 1996a, 1996b) revealed that low permeation of several CPAs could be an important factor in difficulties with teleost egg

cryopreservation. To facilitate the penetration of CPAs, several studies have used dechorionated embryos (Harvey et al. 1983, Zhang and Rawson 1996a, Hagedorn et al. 1997). Although it might simplify cryopreservation by reducing the number of osmotic barriers, for practical purposes it would be beneficial to use eggs with intact chorions. Other studies have shown that the chorion is not a major barrier of water and CPA movements in embryos of zebrafish *Brachydanio rerio* (Zhang and Rawson 1996b).

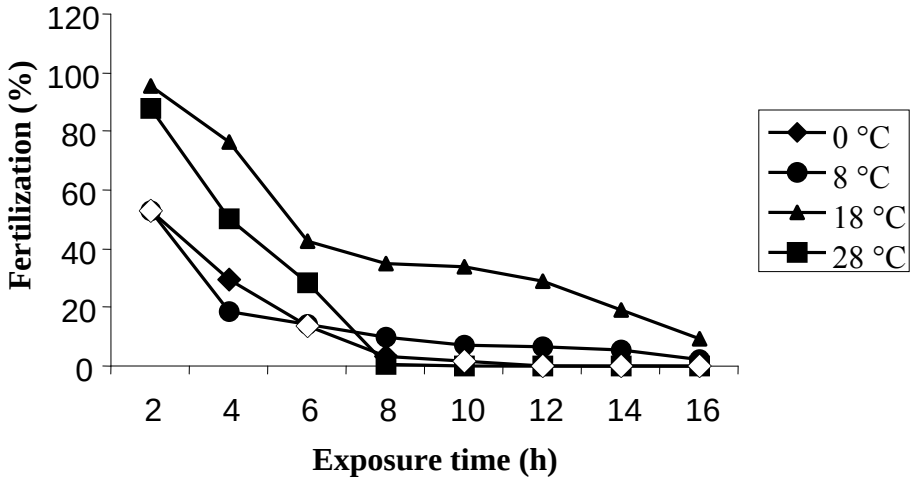

Figure 3. Fertilization of unactivated sharptooth catfish eggs at different storage temperatures.

In our experiments on sharptooth catfish eggs with intact chorions, permeability to water and CPA was tested by a simple method (Dinnyes et al. 1993). Unfertilized eggs and 2-cell, 4-cell, 8-cell, early morula, late blastula, and early gastrula stage embryos (n = 50 per group) were exposed to 6.5 M DMSO for 10 min and 6.5 M glycerol (GLY) for 5 min at 23 °C. Following equilibration, eggs were placed into a drop of 6.5 M GLY and loaded into 0.25-mL plastic straws. Straws were plunged directly into LN_2 and the presence or absence of intracellular ice crystals was monitored by the opacity or transparency of the eggs. Vitrification rate was defined as the percentage of eggs remaining fully transparent in LN_2. Vitrification of the eggs in those cases was probably a combined result of dehydration and CPA penetration (Figure 4). The relatively high permeability of unfertilized eggs decreased rapidly after fertilization and increased again in the morula and later stages. Unactivated eggs were selected for further experiments on CPA toxicity and vitrification, based on their practical importance, moderate chilling sensitivity, and relatively high permeability.

Cryoprotectant Toxicity

Cryoprotectants are considered necessary for the cryopreservation of cells, although toxicity can be a major problem, especially at the high concentrations needed for vitrification. In teleosts the situation is complicated by the fact that the CPA concentration needed for vitrification can be achieved faster in embryonic cells than in the yolk compartment (Hagedorn et al. 1996b). The exposure time needed to reach a sufficient level of CPA in the yolk can be deadly for embryonic cells. The use of unactivated eggs which lack the syncytial layer, simplifies that problem as well. In our experiments, unfertilized eggs were exposed to 6.5 M DMSO, GLY, propylene glycol (PG) or 2,3 butanediol (BD) for 1, 3, 5, 7 or 9 min at 23 °C (n = 200 per group).

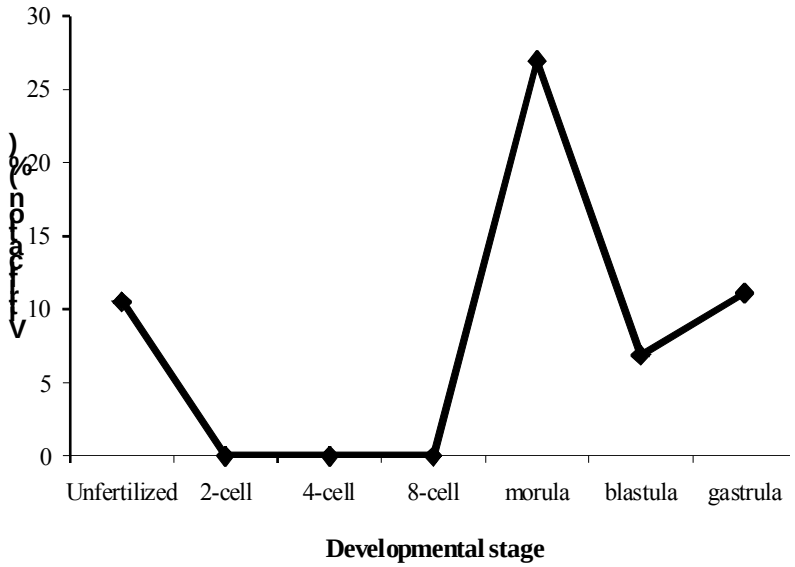

Figure 4. Vitrification of eggs of sharptooth catfish at different developmental stages following equilibration in 6.5 M DMSO and glycerol. Virtification rate was defined as the percentage of the eggs remaining fully transparent in liquid nitrogen (LN_2).

All CPA solutions were prepared in Ringer's solution modified by Ginsburg (1968). Data were analyzed by analysis of variance (SAS 1988).Following two rinses with Ringer's solution, eggs were fertilized and cultured further. Fertilization rate was defined as the percentage of treated eggs developing to the 16 to 32-cell stages. In the control group, fertilization rate was 78% (Figure 5). Glycerol was least toxic followed in order by PG, DMSO and BD. The reasons for the toxicity of different CPAs are not known.

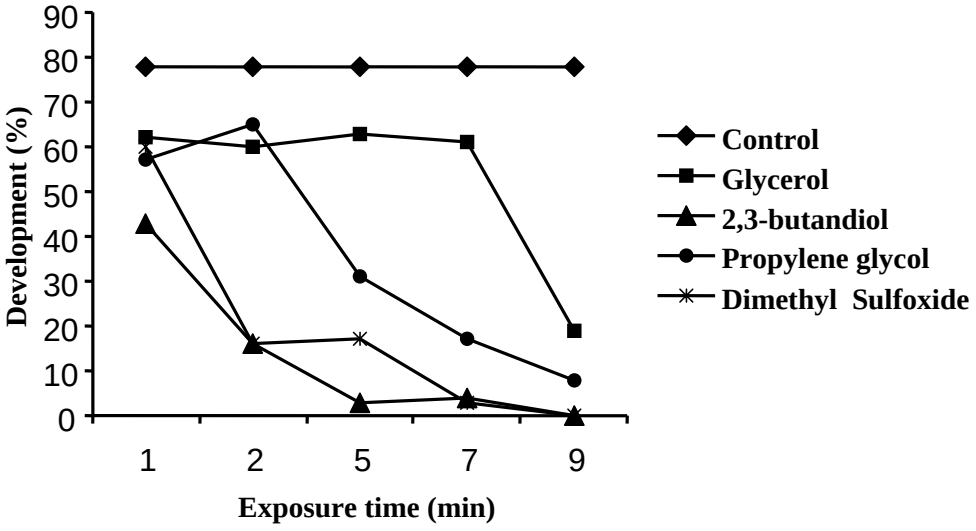

Figure 5. Development to the 8 to 16-cell stage of sharptooth catfish eggs following exposure to cryoprotectants.

Vitrification

Vitrification, an ice-free cryopreservation method, based on the combined effect of high cooling speed and elevated CPA concentrations (Rall and Fahy 1985) offers some advantages compared to traditional freezing methods. It has the potential to overcome problems related to chilling sensitivity as demonstrated in *Drosophila* embryos (Steponkus et al. 1990, Mazur et al. 1992). In teleosts, however, vitrification experiments have failed to result in survival (Zhang and Rawson 1996b).

In our experiments vitrification of unactivated catfish eggs was studied by visualizing the presence or absence of ice crystals following exposure to various CPAs. Unactivated eggs were exposed to 6.5 M BD for 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, 17.5 or 20 min with or without a 1-min pre-treatment in 6.5 M DMSO (n = 100 per group). Following equilibration the eggs were transferred into 6.5 M GLY and loaded into 0.25-mL plastic straws. The straws were plunged into LN_2 and vitrification was observed (Figure 6) as described above. Following 1 d of storage in LN_2 the eggs were thawed in a 40 °C water bath for 8 to 10 sec, rinsed in Ginsburg solution, and were fertilized. Vitrification rate of eggs equilibrated in BD without DMSO pre-treatment was 0% for the first 5 min, but gradually increased with time. Pre-treatment with DMSO increased the vitrification rate to 100% within the first 5 min of exposure to BD. In a toxicity test, exposure to DMSO for 1 min and BD for 2 min without vitrification resulted in 3% fertilization. In most cases, egg morphology was normal immediately after thawing, however, no embryonic development was observed among the thawed and fertilized eggs.

In conclusion, our results indicate that in sharptooth catfish, permeability of embryos decreases sharply after fertilization and remains low until morula stage. High fertilization rates can be achieved following short exposure of unactivated and unfertilized eggs to vitrification concentrations of CPA. A 1-min exposure to DMSO facilitates vitrification with other CPAs, probably by modifying the permeability of the membranes. Other mixtures of CPAs should be tested to decrease the toxicity of the vitrifying solutions. Vitrification is a promising method, although further studies are needed to make it possible for teleost eggs.

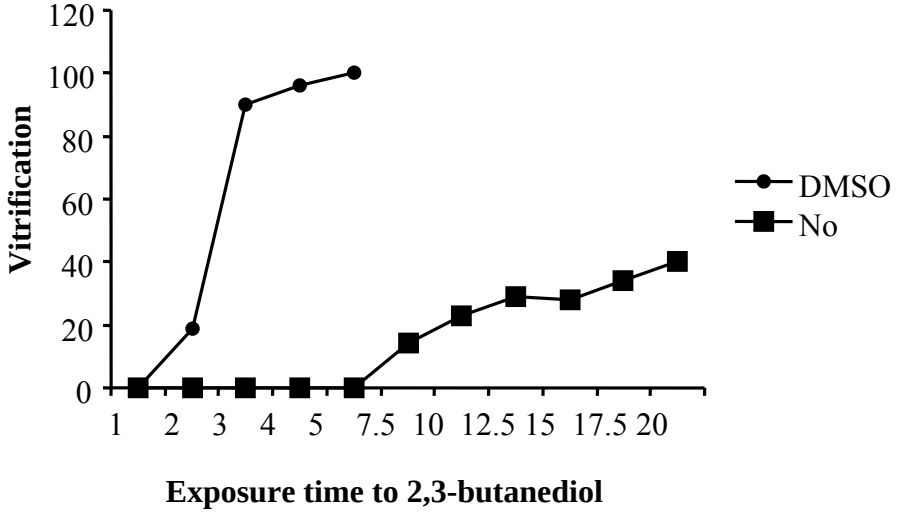

Figure 6. Vitrification of sharptooth catfish eggs exposed to 2,3-butanediol with or without pre-treatment in DMSO.

Freezing of Isolated Blastomers

Gene preservation by the cryopreservation of isolated blastomers has been studied. Freezing of blastomers and embryonic cells isolated from rainbow trout *Oncorhyncus mykiss* (Nilsson and Cloud 1993) and zebrafish (Harvey 1983) embryos seems to be straightforward. According to our preliminary experiments in sharptooth catfish, freezing of blastomers from morula-stage embryos by a two-step method in DMSO or methanol resulted in satisfactory membrane integrity of thawed cells (Magyary et al. 1994). Aggregation of the thawed cells to dechorionated embryos for further chimeric development, however, failed to result in progeny. This method would be far from practical, due to the necessary micro-manipulations, but could be useful for endangered species.

Perspectives of Vitrification of Teleost Eggs

Vitrification of unactivated eggs would allow numerous practical applications. In our experiments the gap between the tolerated and effective vitrifying CPA concentrations and exposure times was reduced using DMSO pretreatment. The practical and efficient detection of ice crystal formation (Dinnyes et al. 1993, Zhang and Rawson 1996b) does not allow determination of whether vitrification is due to dehydration or CPA penetration, and whether it happened through intact or damaged membranes of the eggs. Other methods, mentioned above (i.e. radiotracer and NMRI studies) (Harvey et al. 1983, Magnus et al. 1995, Hagedorn et al. 1997) are more suitable to reveal such details. Those methods, however, are difficult to apply and the data do not show which compartment of the embryos was permeated. Future technical advances probably will allow more insights into the details of CPA penetration into teleost embryos.

Technical advancement is constantly improving vitrification methods. Certain physical factors can facilitate the vitrification of solutions. For example, increases in cooling rate, obtained, through improved heat transfer on cooled copper blocks, resulted in the successful cryopreservation of the large and yolky eggs of *Drosophila* (Steponkus et al. 1990, Mazur et al. 1992). Some other options, like the reduction of sample size are limited by the relatively large size of the teleost egg. Reduction in the atmospheric pressure to increase vitrification, however, has not been reported with teleost eggs. Organ preservation studies are concentrating on these issues, resulting in subtle, but sometimes crucial reductions in CPA concentrations or exposure times. Teleost egg preservation is a difficult cryobiological challenge, which will probably be solved in the near future in some species. Traditional methods of mammalian embryo preservation have failed to provide an easy analogy and solution. Advances in related fields of cryobiological research, especially those dealing with larger samples, such as tissues, organs or complete bodies might provide useful examples for teleost egg cryopreservation.

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Summary of Recent Developments and Advances by Authors

In the past 9 yr since the first edition of this book, relatively few papers have been published on the cryopreservation of sperm of African catfish (Viveiros et al. 2000, 2001, Rurangwa et al. 2001, Miskolczi et al. 2005). There were principally three groups in Europe working on this species that published protocols: one at the University of Wageningen in the Netherlands, one at the Catholic University of Leuven in Belgium and one at Szent István University in Gödöllő, Hungary. The three groups work independently of each other, thus, their protocols differ in several details. The first group used Ginzburg fish ringer as a basic extender to dilute sperm 10× before cryopreservation in 1 ml cryovials with a programmable freezer. The development of cryopreservation protocols, cryoprotectant type, cryoprotectant concentration, cooling rate and sperm-egg dilution ratio (Viveiros et al. 2000) as well as plunging temperature (Viveiros et al. 2001) were optimized. In the first study, best hatch results were observed with sperm that were frozen using slow cooling rates (-5 °C/min or -10 °C/min) to relatively high end

temperatures (-45 °C or -55 °C). Concerning cryoprotectants, best hatch results were found when sperm were frozen in presence of methanol in 10% (v/v) concentration. It was also found that a post-thaw dilution of sperm with the extender at a ratio of 1:200 improved hatch results. In the sec study, it was concluded that sperm has to be cooled below -38°C before plunging into liquid nitrogen when slow cooling rates (-2 °C/min or -5 °C/min) are employed. An isothermal holding period before plunging was also found beneficial for post-thaw sperm survival as it has been indicated in previous studies (Steyn 1993).

The study of Rurangwa et al. (2001) introduced the use of computer-assisted sperm analysis (CASA) into work on cryopreservation of African catfish sperm as a quality control system. Sperm quality was assessed following storage of sperm for 2 d (in case of freshly extracted sperm) or following cryopreserved storage for 2 d and 5 or 10 mo. Glycerol or DMSO were used as cryoprotectants in combination with several extenders. Cryopreservation of sperm was conducted in 1.8 ml cryotubes using a programmable freezer. Sperm quality was assessed by fertilization percentages, motility parameters measured by CASA and viability measured by trypan blue staining of cells. Best cryoprotection was observed using Mounib's solution as cryoprotectant in combination with either 8% DMSO or 5% DMSO and 5% glycerol. Generally viability and motility of sperm correlated with their fertilizing capacity. It was also observed that quality of sperm stored at 4 °C for 2 d was lower than that of cryopreserved sperm after 2 d of storage in liquid nitrogen.

The Hungarian group has investigated the suitability of using straw types of different volumes and the ploidy of deformed larvae occurring during cryopreservation of sperm (Miskolczi et al. 2005). Sperm of African catfish was frozen in a 6% fructose solution using either DMSO or methanol in 10% (v/v) final concentration and a 1:1 final dilution ratio of sperm. Sperm was loaded into straws of different volumes (0.25, 0.5 and 1.2 ml) and frozen in the vapor of liquid nitrogen in a styrofoam box. Fertilization percentages at the 4-8 cell stage, hatch percentages and ratios of deformed larvae were determined. Ploidy levels of deformed larvae were determined using chromosome counting in cell suspensions of individual larvae. Fertilization percentages of 62-83% (83% in the control) and hatch rates of 43-53% (59% in the control) were observed without any significant effect of either straw sizes or cryoprotectants used. The ratio of deformed larvae among all hatched fry was surprisingly high: 33-44%, and even the control group produced 28±13% larval malformation. Ploidy investigations determined that most deformed larvae were diploid thus they were the result of improper egg incubation conditions rather than the genotoxic effect of cryopreservation. Haploid individuals, however, were found among deformed larvae hatched from eggs fertilized with cryopreserved sperm which were absent in the control. Triploid, tetraploid and mosaic individuals were equally found in batches fertilized with cryopreserved sperm and the control. Authors attributed the occurrence of haploid larvae to possible genotoxic effects of freezing or thawing.

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Cryopreservation of Gametes and Embryos of Cyprinid Fishes

István Magyary, Béla Urbányi, Ákos Horváth and András Dinnyés

Aquaculture, a sustainable means of producing large amounts of quality food, is rapidly gaining importance. Several cyprinid fishes are among the most important farmed species in Europe and Asia. Long term preservation of teleost gametes could be highly beneficial for fish farming and wildlife preservation (Rall 1993). Successful freezing of sperm has been achieved in several species (McAndrew et al. 1995) including the common carp *Cyprinus carpio*. However, cryopreservation of teleost embryos has not been accomplished. In the following chapter the cryopreservation of common carp sperm and the chilling sensitivity of embryos in four cyprinid species is discussed.

Cryopreservation of Common Carp Sperm

Cryopreservation of common carp sperm has been extensively studied. In early trials fertility of thawed sperm was either not tested (Sneed and Clemens 1956) or was found to be low (Kossman 1973, Moczarski 1977, Stein and Bayrle 1978). The first practical results were reported by Kurokura et al. (1984). Koi carp sperm were frozen in a methanol-dry ice bath in 0.5-mL straws. Freezing with "Extender 2" and 15% dimethyl sulfoxide (DMSO) resulted in a 69% fertilization rate (fresh sperm control was 83%) following thawing. Cognie et al. (1989) used Tris-buffered diluent containing NaCl, KCl, and 10% DMSO for freezing of common carp sperm in 0.25-mL plastic straws. Although high post-thaw motility (70 to 80%) was achieved and a high percentage (66% of the control) of thawed spermatozoa were intact, the fertilization rate remained low (30 to 40% of the control) when eggs were fertilized in a Tris-buffered solution containing NaCl and glycine. Lubzens et al. (1993) achieved a fertilization rate of 53 ± 6% (control: 80 ± 8 %) and 75 ± 9 % hatching when koi carp sperm was diluted in Kurokura's Extender 2 containing 10% DMSO at 1:5 dilution rate, and frozen at 5 °C per min rate in 1-mL plastic tubes. Eggs were fertilized in water in Petri dishes.

The freezing of fish sperm is affected by several physiological and technical factors. An appropriate oxygen level for the sperm is, possibly, one of the most important factors, which is often compromised by transportation and storage methods. Saad et al. (1988) obtained the best results for short-term preservation of carp sperm when undiluted sperm were stored in thin layers exposed to air or oxygen. According to Billard et al. (1995), this effect of oxygen is possibly due to the recovery of the ATP stores of the spermatozoa. These authors also speculated that recovery of sperm motility before freezing might be a key point for further research. Recovery of the motility potential of carp sperm was studied by Redondo-Muller et al. (1991) after incubation in 200 mM KCl solution, indicating the beneficial effect of potassium ions.

In our own experiments, the goal was to improve survival of common carp sperm by optimizing extenders, dilution and fertilization steps and oxygen concentrations. Some Basic Methods (Magyary, et al., this volume) contains details of an optimized method for freezing of common carp sperm, based on the results discussed below. For the experiments, milt samples derived from different males were transported separately on ice in 2 to 5 mL volumes either in plastic petri dishes (90 mm) or in glass test tubes (10 mL, 10 mm in diameter). Sperm samples of 5 mL were pooled for further studies. Samples with reduced motility upon transportation were

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exposed to oxygen. The pooled samples were stored before freezing on ice in petri dishes. Sperm freezing was carried out within 2 to 4 hr of stripping. Post-thaw motility of sperm was estimated by the percentage of spermatozoa showing progressive movement in water within 10 s.

Three extenders were compared: 'Extender 2' (Kurokura et al. 1984) modified 'Extender 2' (Magyary et al. 1996a), and modified extender (without 'Menezo B2' and urea) (Cognie et al. 1989). Fertilization tests were carried out in Woynarovich solution (Wsol) (Woynarovich 1962), 0.4% NaCl, 0.3% urea or water and were evaluated according to Magyary et al. (1996b). Optimal ratios of thawed sperm, eggs, and water were determined in experiments where the number of spermatozoa per egg varied between 5×10^4 and 3×10^6, and the egg to water volume ratio varied between 5:1 and 1:5. Fertilization rates were recorded at the 8 to 16 cell stage. Embryos were incubated in Zug-jars until hatching. Data were analyzed by the Student's t-test.

The Effect of the Oxygen Addition During Sperm Transportation

The effect of oxygen addition was studied for sperm transported in glass test tubes (Table 1). Sperm transported without supplemental oxygen generally showed reduced motility. Pre-cooling, post-equilibration and post-thaw motility were significantly enhanced by exposure to oxygen. Samples oxygenated for at least 30 min showed significantly higher post-thaw motility than did non-oxygenated samples (Figure 1). Sperm transported and stored in thin layers in Petri dishes showed higher, post-thaw motility (50 to 60%) without any additional oxygen treatment.

Table 1. The effect of oxygen supplementation on motility of fresh, equilibrated and thawed sperm of common carp.

	Percent of Motility (mean ± SD)	
Sperm treatment	**O_2 added**	**No O_2 added**
Fresh	92 ± 9	50 ± 12
Equilibrated	57 ± 10	13 ± 4
Thawed	40 ± 10	8 ± 3

Freezing methods for sperm cryopreservation of common carp are continuously improving. Resistance to cryopreservation of common carp sperm is determined mainly by sperm quality. Sperm quality seems to have a strong relationship to oxygen supply, which is highly influenced by the conditions of transportation and storage (Table 1), and storage in Petri dishes in thin layers on melting ice was found to be superior to transportation in tubes, confirming the observations of Saad et al. (1988). Decreased motility of common carp sperm due to transportation and storage was restored by 30 min of oxygen treatment (Figure 1), probably due to a regeneration process of the spermatozoa ATP supplies (Billard et al. 1995). Oxygen-treated spermatozoa showed enhanced tolerance to equilibration in cryoprotectants and freezing. Further studies are essential in this field to determine the changes in ATP content of spermatozoa exposed to different oxygen levels and the effect on their cryopreservation.

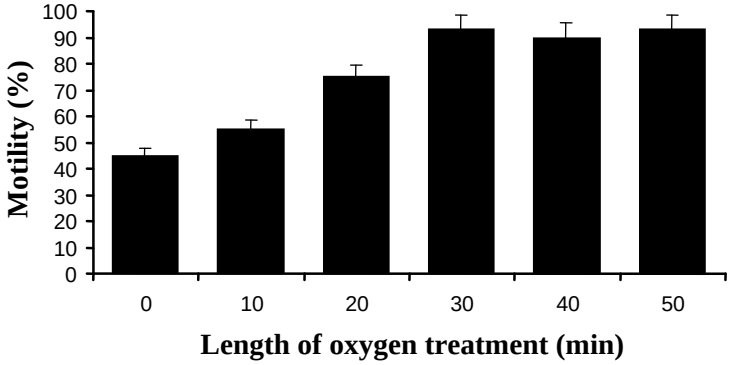

Figure 1. The effect of oxygen treatment prior to freezing on the post-thaw motility (mean ± SD) of common carp sperm.

Comparison of Different Extenders

Significantly higher post-thaw motility was achieved by using modified Extender 2 in comparison with other extenders (Figure 2). The decrease in motility observed during equilibration was probably due to the toxicity of DMSO in the diluent at temperatures above 0 °C. According to our results, the freezing tolerance of common carp sperm was correlated with the motility observed during equilibration, underlying the importance of the motility checks.

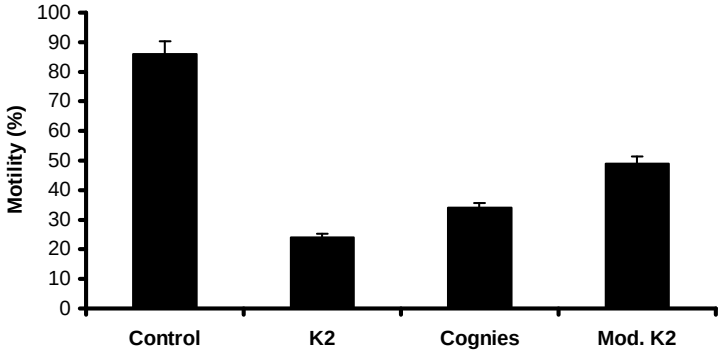

Figure 2. The effects of different extenders on the post-thaw motility (mean ± SD) of common carp sperm. Abbreviations: Control: undiluted fresh milt control; K2: Kurokura's extender 2; Cognies: modified Cognie's extender; Mod. K2: modified Kurokura's extender 2.

In the modified Extender 2 the concentration of potassium ions was higher and that of sodium ions was lower compared to the original composition. In comparison with the original Extender 2 and the extender described by Cognie et al. (1989) those modifications were beneficial, resulting in significantly higher motility after thawing. These differences might be due to the increased regenerating effect of potassium ions, reported by Redondo-Muller et al. (1991).

Sperm to Egg Ratios and Diluting Solutions for Fertilization

The optimal ratio of thawed sperm to eggs, sufficient to reach a maximum rate of fertilization, was found to be 1 to 1.5×10^5 sperm per egg (data not shown). Higher ratios were found to decrease the fertilization rates possibly due to the higher amount of toxic DMSO added with the increased amount of thawed sperm.

Using this sperm concentration, steps of the fertilization procedure were studied. Woynarovich solution is generally used in fish farming for fertilization and to reduce the stickiness of fertilized carp eggs, allowing mass incubation and hatching. When first sperm, and then Wsol were added to dry eggs, the fertilization rates were low with thawed sperm (4 ± 3%) and significantly higher (93 ± 5%) using fresh sperm. Similar rates were achieved by replacing the Wsol with 0.4% NaCl (5 ± 4%) or 0.3% urea solutions (6 ± 4%). Both Wsol and its components were found to drastically reduce the fertilization rate with thawed common carp sperm, while fresh sperm was able to fertilize the eggs at high rates in all of these solutions. Addition of water, however, significantly improved fertilization rates with thawed sperm (89 ± 6%).

Moreover, the order of addition of thawed sperm and water to eggs was also found to have a significant effect on fertilization rates (Figure 3). When first Wsol or water was added to the eggs and 5 sec later the thawed sperm, significantly higher fertilization rates were achieved when compared to adding the sperm before the solutions. Using the best method, water followed by thawed sperm, fertilization (95% of control) and hatching (95% of control) rates were not significantly different from those of the non-frozen sperm control. After fertilization (1 min), the water was replaced with Wsol in order to avoid stickiness of eggs. The optimal ratio between eggs and water was found to be 1 to 1 in volume. The best fertilization rate (100% of control) was achieved with a 1:20:20 volume ratio of thawed sperm, eggs and water.

In conclusion, technical improvements in sperm freezing methods resulted in improved fertilization and hatching rates in common carp, not different from those of control treatment. Furthermore, improved results were achieved in a study on sperm cryopreservation of koi carp and goldfish *Carassius auratus* sperm applying similar technical modifications (Bercsenyi et al. 1998).

These improved methods for cryopreservation, thawing and use of common carp sperm, are suitable for genome banking in common carp, allowing the preservation of valuable stocks and enhanced efficacy of commercial breeding efforts.

Chilling Sensitivity of Cyprinid Embryos

Cryopreservation of teleost embryos is a major cryobiological challenge. The difficulties of teleost embryos could be related to chilling sensitivity, or to structural characteristics like the large yolk compartment, chorion layer, and low permeability of the membranes (Hagedorn et al. 1996, Hagedorn et al. 1997, Rall 1993). Chilling sensitivity (i.e. specific sensitivity to low temperature that is not related to ice crystal formation) has been reported in *Drosophila* embryos (Mazur et al. 1992, Steponkus et al. 1990), bovine oocytes (Arav et al. 1996), pig oocytes and embryos (Nagashima et al. 1992, Dobrinsky et al. 1995), *in vitro*-produced bovine embryos (Leibo and Loskutoff

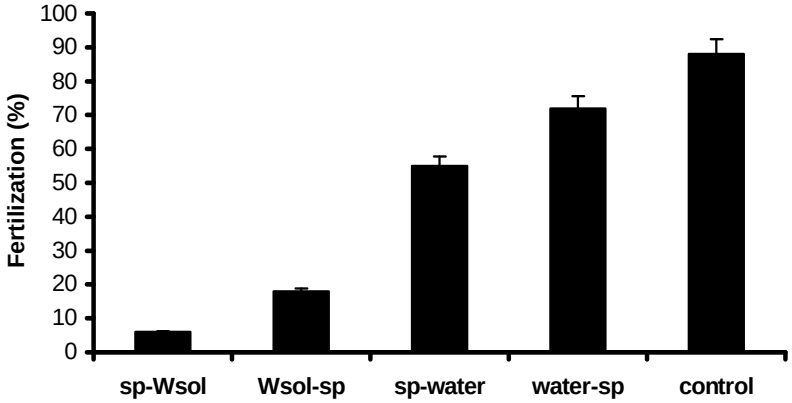

Figure 3. The effect on fertilization rates of the order in which different solutions and sperm were added to the eggs of common carp. Abbreviations: sp-Wsol, sperm followed by Woynarovich solution added to eggs; Wsol-sp, Wsol sperm added to eggs; sp-water, sperm followed by water added to eggs; water-sp, water followed by sperm added to eggs; control, fresh sperm followed by Wsol added to eggs.

1993) and in embryos of some teleost species including zebrafish D*anio rerio* (Zhang and Rawson 1995, Hagedorn et al. 1996, 1997), red sea bream *Purgus major*, olive flounder *Paralichthys olivaceu*, multicolor rainbowfish *Melanotaenia affinis* (Sasaki et al. 1988), red drum *Sciaenopus ocellatus* (Gwo at al. 1995), goldfish (Liu et al. 1993) and African catfish *Clarias gariepinus* (Dinnyes et al. 1996).

Low temperature can be detrimental to cells. Chilling sensitivity is the result of direct and indirect chilling injuries. The extent of the damage is related to the cooling rate, the temperature and the time for which cells are exposed to low temperature. Several theories exist for the mechanism of chilling sensitivity, but they probably differ by species and cell type. At low temperatures microtubules are depolymerized (Magistrini and Szöllösi 1980) and cellular processes such as cell division in oocytes can be irreversibly disrupted (Magistrini and Szöllösi 1980, Martino et al. 1996). The plasma membrane can suffer lateral phase separation (Arav et al. 1996); proteins can be denatured due to the destabilization of hydrophobic bonds (Brandts 1964); the cell membrane can shrink relative to the intracellular space which may result in stresses and damage to the membrane (McGrath 1987), and disruption of metabolic and enzymatic processes due to their inhibition at sub-physiological temperatures can occur (Mazur et al. 1992). These can be especially detrimental in fast-developing embryos like *Drosophila* or some teleosts.

There are some data on chilling sensitivity and the effect of cryoprotectants (CPAs) in common carp embryos (Jaoul and Roubaud 1982, Magnus et al. 1995, Roubaud et al. 1985, Zhang et al. 1989, Dinnyes et al. 1998) and non-fertilized eggs (Urbanyi et al. 1998), however, no data are available for other cyprinid species. Differences among closely related species are not well documented. The following study was aimed at assessing and comparing the sensitivity of embryos of four farmed cyprinid species to cryoprotectants and low temperatures at different developmental stages.

Methods to Study Chilling Sensitivity and Cryoprotectant Toxicity

Eggs of common carp, silver carp *Hypophthalmichthys molitrix,* bighead carp *Aristichtys nobilis* and grass carp *Ctenopharyngodon idella* were obtained. In a series of experiments, eggs from one female were mixed with sperm of two males and were used in each treatment group. In the four species a total of 31,800 embryos in the developmental stages of morula, half-epiboly and heart-beat were treated. Experiments were performed in triplicate and each treatment group consisted a total of 900 embryos. In the experiments on chilling sensitivity, embryos in water were placed into a waterbath at 0 to 0.1 °C for 1 hr. In the experiments on the toxicity and protective effects of in 1 M DMSO, methanol, and glycerol and 0.1 M sucrose solution, embryos were placed in a waterbath at 0 °C for 1 hr. Data were analyzed using a randomized complete block analysis of variance via the SAS GLM procedure (SAS 1988). For analyses, percent survival values were arc-sine transformed. Significant differences were evaluated using Fisher`s LSD analysis of the transformed data.

Effect of Mechanical Manipulation and Chilling of Embryos

Temperature, duration, developmental stage, cryoprotectants and all interactions had significant effects on survival ($P < 0.001$). To compare the results for the different species, hatching rates were expressed as a percent of the hatching rates of control treatments at room temperature. Common carp embryos were extremely sensitive in morula and half-epiboly stages; in heartbeat-stage, bighead embryos were particularly sensitive although in other species that stage was more tolerant (Figure 4).

Our results with the room temperature control groups (Table 2) showed that in species with floating eggs, half-epiboly was significantly more sensitive to the mechanical stress of the treatments than were heartbeat and morula stages. Embryos from common carp, a species with demersal eggs, were less sensitive to the mechanical effects of embryo manipulation. However, in species with floating eggs, the drop in half-epiboly stage clearly represented a stage-dependent increase in sensitivity to mechanical stress. The morulae of red drum species were more sensitive to handling than were half-epiboly and heart-beat stages (Gwo et al. 1995). In red drum, the developmental stages most sensitive to mechanical stress were least sensitive to chilling. In grass carp there was a similar tendency, but more specific studies are needed to examine the relationship between these two kinds of sensitivity. In the other three species this pattern was not found. The stage-dependent sensitivity to a relatively minor mechanical stress was an important factor during treatments in embryos of bighead carp, silver carp and grass carp.

Our results of low temperature exposures indicate that cyprinid embryos were sensitive to chilling. In every species and at every developmental stage a significant decrease in hatching rates was found following exposure to 0 °C. The sensitivity in common carp was higher than in the other carp species or in some other teleosts (Whittingham and Rosenthal 1978, Zell 1978). The reasons for the extent of this chilling sensitivity might be related to the changes in cell and tissue types, numbers of cells, effectiveness of repair mechanisms and enzymatic reactions. Changes in the size and structure of the yolk compartment and membrane structures might be among the key factors. At the heart-beat stage, the functioning blood circulation could prevent or limit injuries and increase the efficiency of repair mechanisms. It has been suggested that the progressive acquisition of tolerance to cold shock in common carp embryos may be related to the slowing down and desynchronization of cell division cycles at later stages (Roubaud et al. 1985). Our finding that more advanced embryos exhibited a higher tolerance towards low temperatures

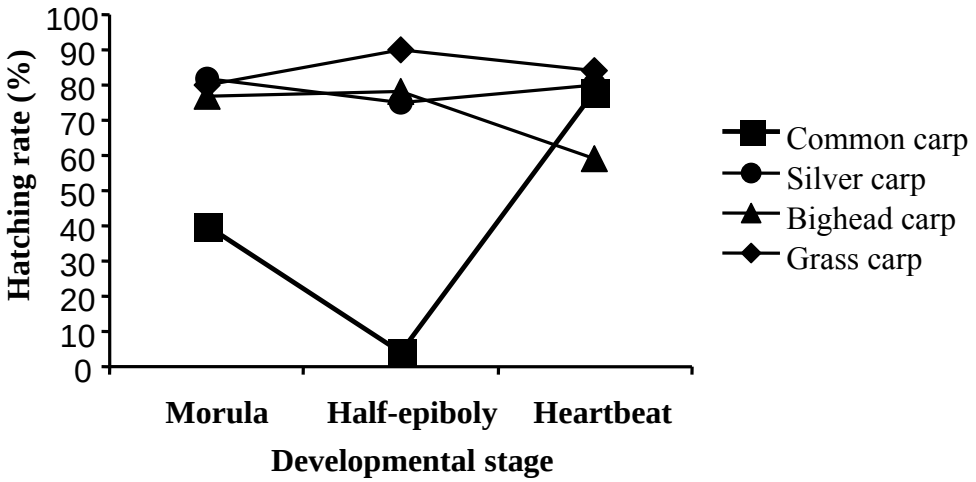

Figure 4. Chilling sensitivity of cyprinid embryos following 1 hr at 0 °C at three developmental stages.

is similar to that reported elsewhere for common carp (Jaoul and Roubaud 1982, Roubaud et al. 1985, Magnus et al. 1995, Dinnyes et al. 1998) and other species (Sasaki et al. 1988, Liu et al. 1993, Hagedorn et al. 1996). In contrast to the common carp data, the opposite order of chilling sensitivity in morula, half-epiboly and heartbeat stages were reported for red drum embryos (Gwo et al. 1995). Also, we have found heartbeat stage bighead carp embryos were more sensitive than were earlier stages. The key factors of chilling sensitivity in the different stages are not known, and obviously even closely related species (i.e. bighead carp and silver carp) can be diverse in that sense. However, from a practical point of view, knowledge of the most resistant stages will assist in the design of cryopreservation protocols.

Table 2. Hatching rates (mean percent) of various cyprinid embryos following culture at 24 °C.

	Developmental stage		
Common name	**Morula**	**Half-epiboly**	**Heartbeat**
Common carp	95 [a]	94 [a]	91 [b]
Silver carp	81 [a]	67 [b]	74 [c]
Bighead carp	73 [a]	58 [b]	64 [c]
Grass carp	84 [a]	73 [b]	79 [c]

Groups with different superscripts within rows were significantly different ($P < 0.05$). 900 embryos were in each treatment group.

The Toxic and Protective Effects of Cryoprotectants During Chilling

Cryoprotectants modified the survival rates of cyprinid embryos at 0 °C (Figures 5 and 6). Methanol protected carp embryos in half-epiboly stage, but decreased the survival in other species. Dimethyl sulfoxide was toxic to silver carp and bighead morulae and half-epiboly stages, however, it was beneficial for those same stages in common carp. The effect of glycerol and sucrose was similar to the patterns found for methanol and DMSO.

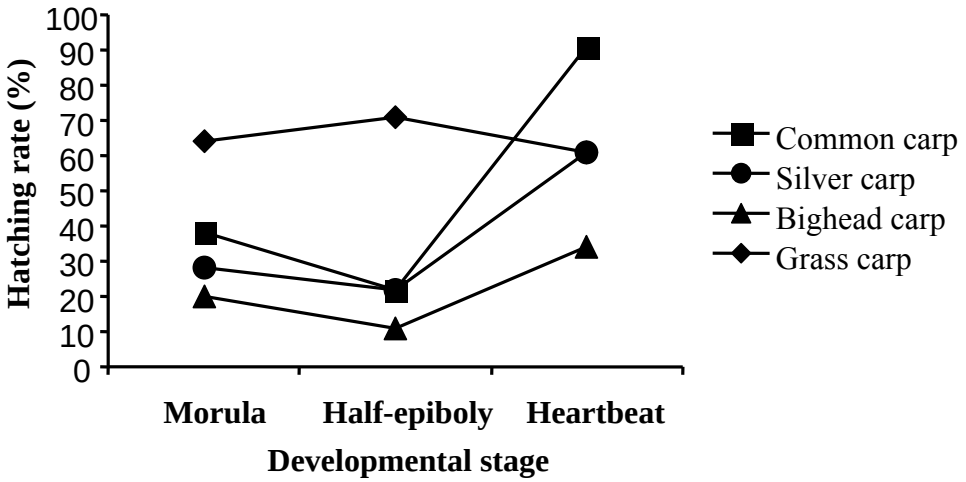

Figure 5. Effect of 1M methanol exposure for 1 hr at 0 °C for cyprinid embryos at three developmental stages.

In common carp, DMSO and sucrose exhibited a significant protective effect against chilling injury for half-epiboly stage at 0 °C, and a limited beneficial effect of methanol and glycerol for half-epiboly and heartbeat stages was detected as well. A stage-dependent tolerance during cooling of common carp embryos to -5 °C in the presence of 2 M DMSO was reported (Zhang et al. 1989). The mechanism by which certain cryoprotectants can effect chilling injuries in embryos is not clear. Some CPAs may protect membrane and sensitive enzymes from low temperatures (Leibo 1981, Schneider and Mazur 1984). Sugars have been reported to protect the membranes from the consequences of dehydration in mammalian embryos (Anchordoguy et al. 1987). The exact reasons for these findings requires further study.

Our results showed that the cryoprotectants tested were toxic to embryos of silver carp, bighead carp, and grass carp at 0 °C. The sensitivity to a given CPA was dependent on species and developmental stage. Probably the hatching rates reflected a balance between the toxic and the protective effects. Our results on the stage dependence of the toxicity are in agreement with findings in zebrafish (Zhang et al. 1993), red drum (Robertson et al. 1988) and common carp (Dinnyes et al. 1998). Cryoprotectant toxicity and protective effects might be related to permeation differences in the different compartments of the embryos, because the chorion, cell membrane and yolk syncytial layer represent different osmotic and permeability barriers and divide teleost embryos into several compartments with different permeability parameters. Mammalian embryology provides possible explanations of mechanisms of CPA toxicity on fish embryonic cells. The cytoskeleton and cell division can be disrupted by some CPAs. Interactions of DMSO with actin filaments (Sanger et al. 1980) in oocytes reversibly depolymerize the cell spindle (Vincent et al. 1989) disturbing cell division. The toxicity of DMSO is highly reduced at lower temperatures (37 ^{o}C vs. 4 ^{o}C) (Pickering et al. 1991). Explanation for the stage dependent sensitivity towards CPA in teleost embryos requires further studies.

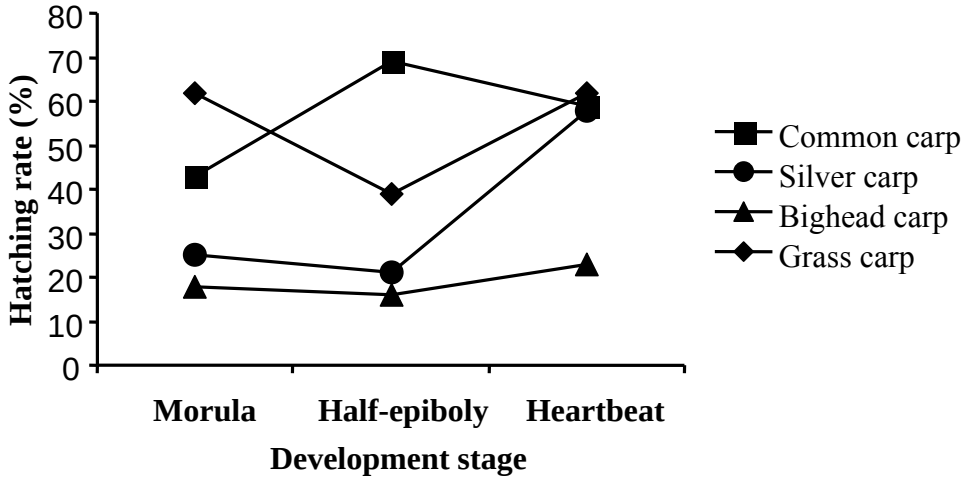

Figure 6. Effect of 1 M DMSO exposure for 1 hr at 0 °C for cyprinid embryos at three developmental stages.

In conclusion, findings on the lower sensitivity to chilling of some developmental stages and the protective effects of certain CPAs can be important for designing cryopreservation protocols. The high variability of toxicity and sensitivity parameters among species and developmental stages indicates the usefulness of empirical studies with different fish species. Further basic studies are needed for a better understanding of the biological mechanisms for low temperature sensitivity.

Acknowledgments

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Summary of Recent Developments and Advances by Authors

Since the publication of the first edition of this book cryopreservation of common carp sperm largely remained within the sphere of interest of Central European research groups. Lahnsteiner et al. (2000) conducted studies on the cryopreservation of sperm from several cyprinid species including common carp using computer-assisted sperm analysis (CASA) to monitor post-thaw sperm survival. Sperm of the bleak *Chalcalburnus chalcoides* was used as a model for the development of a cryopreservation protocol that was later tested on sperm of other cyprinid species. A series of complicated experiments were carried out in order to determine the optimal cryopreservation conditions that included tests on the effects of penetrating and non-penetrating cryoprotectants on pre-freeze and post-thaw motility. Investigations of freezing and thawing conditions such as distance from the surface of liquid nitrogen, thawing temperature and duration were also carried out. In the final protocol, sperm was diluted with an extender labeled "buffered sperm motility-inhabiting saline solution" (BSMIS – 75 mM NaCl, 70 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris, pH 8.0) with a final concentration of 1.0 - 2.5 × 10^9 spermatozoa per ml. Glycine (0.5%) and DMSO (10%) were used as cryoprotectants. Sperm was frozen in 0.5-ml French straws in the vapor of liquid nitrogen poured into an insulated box. Ideal conditions of freezing and thawing were: freezing 4-5 cm above the level of liquid nitrogen (4 cm for common carp sperm), thawing at 25 °C for 30-45 sec (15 sec for common carp sperm). Using this method, 51 ± 7% motile cells were observed with an average velocity of 70 μm/sec. A similar study later by the same group (Lahnsteiner et al. 2003) concentrated on fertilization techniques and media, straw volumes and optimal semen volume for cryopreservation. As in the previous study, the bleak was used as a model species and the developed protocol was tested on other cyprinids. Wet fertilization (distribution of eggs in a fertilization medium and addition of sperm within 10 sec) resulted in low hatch percentages using cryopreserved sperm (unlike in the control). The use of dry fertilization (mixing of eggs and sperm, addition of the fertilization medium within 5 sec) or prolonged sperm-egg contact (mixing of eggs and sperm, addition of the fertilization medium after 30 sec) significantly improved hatch results. It was also observed that in spite of a wide variety of fertilization media tested, hatchery water (chemical composition

unknown) yielded the highest percentage of hatching embryos (75 ± 2%). In contrast, when post-thaw motility was investigated, all fertilization media (acting as activation media this time) produced much higher motility percentages than hatchery water (39 ± 9% in 50 mM KCl, 1 mM $MgSO_4$ medium vs. 13 ± 4% in hatchery water). The use of 1.2-ml straws yielded hatch rates equal to 0.5-ml straws when they were frozen 3 cm above the level of liquid nitrogen and thawed at 35 °C for 20 sec. When this method was applied to the sperm of common carp, 55 ± 12% hatching was observed with 0.5 ml straws and 60 ± 2% with 1.2-ml straws (57 ± 18% in the control).

In the Czech Republic, Linhart et al. (2000) investigated motility and hatching success of eggs fertilized with cryopreserved sperm of the common carp. Sperm was diluted in Kurokura's solution (Kurokura et al. 1984) using 10% DMSO as cryoprotectant, loaded into 2-ml cryotubes and frozen in a programmable freezer at a rate of 4 °C per min from 4 °C to -9 °C and then at 11 °C per min from -9 °C to -80 °C. Samples were thawed at 35 °C for 110 sec. Post-thaw sperm quality was evaluated for motility percentage and velocity using image analysis, percentage of fertilization at gastrulation and hatching as well as larval malformation. Results have shown that although motility parameters (percentage and velocity) were greatly reduced in cryopreserved samples compared to fresh sperm (with differences among individuals) hatch rates and ratios of malformed larvae were similar to the controls.

The effect of dimethyl-acetamide (DMA) as cryoprotectant on motility and fertilizing capacity of common carp sperm was investigated by Warnecke and Pluta (2003). Sperm was frozen in 0.25-ml straws and thawed at 40 °C for 3 sec. Through a series of optimization tests the effects of 11 different cryo-diluents, three freezing profiles and methods (programmable freezer vs. liquid nitrogen) were assessed on the post-thaw motility (measured by CASA) and fertilization percentages. Best post-thaw motility (40 ± 6%) was observed with cryo-diluents labeled Cryo3 based on modified Kurokura's extender 2 (MK2, Magyary et al. 1996a) combined with 200 mM sucrose and 15% DMA and Cryo10 (MK2, 200 mM trehalose, 20% DMA). Sperm frozen in Cryo10 and stored in LN_2 for 6 d resulted in a hatch percentage of 80 ± 2% while sperm cryopreserved in Cryo3 and stored for 349 d yielded only 41 ± 4% hatch and 38 ± 5% swim-up larvae. Authors did not provide an explanation for the low hatch percentages after nearly a yr of storage in LN_2, although the two hatch rates (after 6 d or 349 d of storage) were not comparable due to differences between cryo-diluents used.

Horváth et al. (2003) has conducted a comparison of five extenders (simple buffered solutions of either sucrose, glucose, fructose or KCl and MK2) and two cryoprotectants (10% DMSO or methanol) to investigate their effect on post-thaw motility, fertilization and hatch percentages of cryopreserved common carp sperm. Sperm was diluted 1:9 in the extender and frozen in 0.5-ml straws suspended on a 3 cm high styrofoam frame floating on the surface of liquid nitrogen. Best results were observed using the combination of 350 glucose, 30 mM Tris (pH 8.0) and 10% methanol. Generally, the use of sugar-base extenders and cryoprotectant methanol resulted in higher post-thaw motility and fertilization percentages than ionic extenders and DMSO. Agglutination of sperm was observed in all groups frozen in sugar-based extenders, however, this did not reduce the fertilizing capacity of cryopreserved sperm. The combination of extender and cryoprotectant developed in this study was later used by the same group (Horváth et al. 2007) to test the suitability of using 1.2-ml and 5-ml straws for the cryopreservation of common carp sperm. Sperm quality was assessed as hatch percentage and larval malformation. Ideal conditions of freezing (cooling time) were determined experimentally for both straw types, 4 min for 1.2-ml straws and 5 min for 5-ml straws. Further experiments were conducted to find

the optimal volume of eggs that can be fertilized with one straw of cryopreserved sperm (using the 10 g of egg per one 0.5-ml straw dose determined in the previous study as a standard). The highest percentage of hatched larvae was found when 10 g of eggs were fertilized with the contents of one 1.2-ml straw (86 ± 12%) or 80 g of eggs were fertilized with one 5-ml straw (65 ± 18%) of sperm. Larval malformations were generally low (between 0 and 13%), and surprisingly, the highest number of deformed larvae was observed in the control (15 ± 9%). Ploidy levels of malformed larvae were determined by chromosome counting to investigate possible genotoxic effects of cryopreservation. Although haploid larvae were detected in a very low percentage (2 – 6% of malformed larvae), the majority of malformed larvae were diploids, thus, their malformation cannot be attributed to the effects of cryopreservation. Haploids, however, were found only in batches fertilized with cryopreserved sperm, and were completely absent from the control. Authors speculated that haploids occurred as a result of damage to the genome of spermatozoa during cryopreservation.

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Cryopreservation of Eggs and Embryos from Aquatic Organisms

Jin-Chywan Gwo

Introduction

Cryopreservation has become a standard method for the long-term maintenance of a large variety of cells. This technique has great practical value for *ex situ* breeding programs. Genetic material from individuals crucial to a population can be preserved and used as needed in the future. Cryopreservation can reduce the risks of random catastrophe to small and fragmented populations. The storage of viable gametes and embryos is also potentially useful in aquaculture production management. It would remove the dependence on breeding seasons, increase gene pools, enable impracticable crosses to be made, maintain genetic information for long periods and serve in genetic studies (Stoss 1983). Cryopreserved gametes and embryos can also be used to standardize bioassays of polluted water samples (Grout et al. 1992).

Cryopreservation is now possible for sperm from numerous aquatic species, but is not available for eggs and embryos (Rana 1995, Gwo 1995, Gwo and Lin 1998), except for trochophore larvae of the eastern oyster *Crassostrea virginica* (Paniagua et al. 1998, Paniagua-Chavez, this volume). Compared to eggs, sperm cells are simpler to preserve because of their large numbers, the ease and repeatability of collection and their suitability for cryopreservation. The successful cryopreservation of mammalian eggs and embryos relies heavily on the theoretical concepts of cryobiology. The critical factors involved in cryopreservation studies include: 1) cell size; 2) cell permeability; 3) choice and concentration of cryoprotectants; 4) rates of freezing and thawing, and 5) final storage temperature. A number of specific criteria are of importance in cryopreservation of eggs and embryos: 1) developmental stage; 2) collection media; 3) cryoprotectant; 4) cooling rate; 5) ice nucleation and seeding, and 6) thawing rate (Mazur 1970, Leibo and Mazur 1978). Because of complex interactions among these factors, an optimal cryopreservation protocol developed for one species is not always applicable to eggs and embryos of other species. Neither the cryopreservation protocols of mammalian embryos nor their modifications have achieved successful results with aquatic organisms (Harvey 1983a, Robertson 1986, Robertson et al. 1988, Rall 1993, Subramoniam and Newton 1993, Zhang et al. 1993).

Cryopreservation of Eggs and Embryos of Aquatic Invertebrates

Controlled Slow-Freezing

The stage of development appears to be a critical factor for survival of embryos. Emphasis on cryopreservation has focused on unfertilized eggs, fertilized eggs and 2 to 4 cell stages in the Pacific oyster *Crassostrea gigas* or eggs and adults of rotifers (Koehler 1967, Euteneuer et al. 1984, Bedding et al. 1987, Chen et al. 1989, Lin et al. 1994, Gwo 1995). Little progress in freezing of eggs has been reported. A few (< 0.001 to 0.005%) Pacific oyster eggs were fertilized after thawing and developed into D-larvae on two occasions in seven trials (Naidenko 1997). Eggs and embryos of penaeid shrimp have been difficult to freeze and to date no success has been reported. Lately it has been found that there is increased tolerance to low temperature as compaction and blastulation occur and interest is growing in cryopreservation of

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later embryonic stages (morula, gastrula and trochophore) in the Pacific oyster, trochophore in blue mussel *Mytilus edulis*, pluteus in sea urchin species, nauplii in barnacle species and zoea in penaeoid shrimp (Gakhova et al. 1991, Toledo et al. 1991, Kurokura et al. 1992, Gwo 1995, Anil et al. 1997, Arun and Subramoniam 1997, Barros et al. 1996, 1997, Gwo and Lin 1998, Khin et al. 1998). Although D-stage larvae of Pacific oysters (24 hr after fertilization) revived and swam after thawing, they all had signs of damage to the digestive tract and only 1% of them were still motile after 1 d (Naidenko 1997).

Two major categories of cryoprotectants, penetrating and non-penetrating, are often used in embryo freezing. The cryoprotectants used for freezing of aquatic invertebrates usually penetrate cells. Often, dimethyl sulfoxide (DMSO) has been the cryoprotectant of choice. The type of cryoprotectant used and optimal molarity differs from one species to another. Dimethyl sulfoxide (1.5 to 3 M) has yielded successful results for freezing of barnacle embryos, while propylene glycerol (PG) at 10% (1.3 M) has been used successfully with oyster embryos (Gakhova et al. 1990, Gwo 1995, Anil et al. 1997, Khin et al. 1998). Higher survival rates of nauplii of the barnacle *Balanus amphitrite* were observed when using ethylene glycerol (EG) rather than DMSO or glycerol (GLY) (Anil et al. 1997). It was noted EG was a better cryoprotectant than DMSO or polyvinyl pyrrolidone (PVP) for embryos of sea urchin species (Asahina and Takahashi 1978, 1979). After examining the effect of six different cryoprotectants on 2-cell to 4-cell stage embryos of Pacific oyster and King scallop *Pecten maximus*, it was concluded that the order of cryoprotectant toxicity was methanol, EG, PG, DMSO, GLY and sucrose, with methanol being the least toxic (Renard and Cochard 1989). Dimethyl sulfoxide was shown to be less toxic than acetamide (A), PG and EG for Pacific oyster embryos at eight different embryonic stages (Chao et al. 1994). However, PG appeared to be less toxic than DMSO, EG and GLY for Pacific oysters at the trochophore stage (Gwo 1995). Mixtures of cryoprotectants were used in vitrification of Pacific oyster embryos (Chao et al. 1997). None of the cryoprotectants investigated could be used at concentrations of higher than 4 M.

To avoid excessive toxicity and osmotic stress of the cryoprotectant, embryos are often equilibrated in a stepwise manner. The method of addition of the cryoprotectant has been studied. Initially, the cryoprotectant was added in four or five steps involving 20 to 30 min for DMSO penetration before cooling and freezing for sea urchin (Asahina and Takahashi 1978), barnacle (Gakhova et al. 1991), Pacific oyster (Chao et al. 1997 and Naidenko 1997) and shrimp *Penaeus indicus* (Subramoniam and Newton 1993). Later, survival of Pacific oyster embryos was compared when the cryoprotectant was added in one-step or stepwise procedures and it was observed that one-step addition was beneficial (Chao et al. 1997). Barnacle nauplii could be cooled to -40 °C and thawed without a requirement for stepwise addition of the cryoprotectant (Anil et al. 1997). The one-step addition facilitated and accelerated the freezing process, which was important for field application. The use of reduced salinity seawater (20 to 25 ppt) for dilution of thawed oyster embryos was recommended (Naidenko 1997).

The most widely used extender has been seawater. The addition of saccharide to the medium before freezing may improve embryonic development after thawing of the Pacific oyster and hard clam *Meretrix lusoria* (Renard 1991, Chao et al. 1997). Trehalose has been shown to reduce the toxicity of cryoprotectants (Chao et al. 1994). Addition of 0.22 M trehalose to 1.5 M DMSO decreased the toxicity of DMSO for Pacific oyster larvae particularly when equilibration was carried out at low temperatures (Naidenko 1997). It also has been suggested that the inclusion of trehalose (0.06 to 0.22 M) or glucose (0.22 M) in combination with 1 to 2 M DMSO increased survival of thawed Pacific oyster embryos (Chao et al. 1997, Naidenko 1997).

Dehydration of the Pacific oyster embryos also may be achieved before cooling by using external cryoprotectants which creates a hyperosomotic environment. Pre-treatment with the antioxidant echinochrome A and antifreeze protein (AFP 1) had no significant beneficial effect on the success of freezing eggs, embryos or larvae of the Pacific oyster (Naidenko 1997).

Most embryos are frozen in 0.25-mL straws. Based on the observation that embryos have a low permeability to cryoprotectants, embryos are cooled slowly at rates below -3 °C per min (Mazur 1970). A slow freezing, two-step method was developed (Mazur 1970, Leibo and Mazur 1978). The embryos were plunged in LN_2 at temperatures ranging from -20 to -50 °C, obtaining extensive dehydration. Plunging the embryos between -30 to -40 °C apparently represented a good balance between dehydration and intracellular ice formation and resulted in high survival rates. Thirty percent survival was reported with barnacle embryos that were frozen in 3 M EG, cooled from 20 °C to 0 °C at -5 °C per min, at -1 °C per min to -8 °C, and at -0.3 °C per min to -40 °C before plunging into LN_2 (Anil et al. 1997). No survival was found when plunging occurred at other temperatures (-20, -30, or -50 °C). More Pacific oyster embryos survived when cooled at -1.5 °C per min to -30 °C before plunging than did when plunged at -10 or -40 °C (Gwo 1995). It has been shown that blue mussel embryos transferred at -30 °C had better survival than did embryos transferred at -40 °C (Toledo et al. 1989). Incorporation of an isothermal holding period in the freezing program led to a marked improvement in embryo survival. Barnacle embryos cooled to -5 °C, seeded and transferred to -30 °C for 20 min before plunging in LN_2 yielded better survival rates than did those obtained by direct transfer to LN_2 (Khin et al. 1997, 1998).

There is a wide range of optimal parameters for freezing of Pacific oyster embryos. Variation in the type and concentration of cryoprotectant and in freezing procedures suitable to obtain viable embryos after thawing included 1.3 M of PG (Gwo 1995) to 1 to 3 M DMSO (Rana et al. 1992, Naidenko 1997). Similar results were found with 2 M and 3 M DMSO, and there was limited variation in groups exposed to rates of -2, -2.5, -3, -4 and -5 °C per min for freezing of Pacific oyster embryos (Chao et al. 1997). Apart from the freezing technology itself, survival rates after thawing can be also affected by embryo quality (Toledo 1990, Gwo 1995).

Vitrification

A 15% survival rate was reported with the embryos of Pacific oyster frozen using a vitrification method (Chao et al. 1997). The embryos were exposed for 15 min at room temperature to a solution of 2 M DMSO in sea water, and then to a solution of 3 M DMSO plus 1 M EG in sea water for 2 min at 0 °C. After transfer to a final vitrification solution containing 5 M DMSO and 3 M EG plus 6% polyvinyl pyrrolidine (PVP) in sea water at 0 °C the embryos were drawn into straws within 30 sec and plunged into LN_2. The authors indicated a stage-dependent sensitivity of the embryos to the vitrification method and observed that the presence of sucrose in the devitrification procedures yielded higher survival rates.

Cryopreservation of Fish Eggs and Embryos

Controlled Slow-freezing

Although a few papers have claimed successful cryopreservation of fish embryos (Wang et al. 1987, Chen et al. 1988, Zhang et al. 1989, Leung and Jamieson 1991, Zhang et al. 1994, 1996, Zhang et al. 1997), there are no reproducible cryopreservation protocols (Rana 1995). The large egg size (0.95 mm diameter in many marine fishes and 6 mm in salmonids), low cell

permeability, sensitivity to chilling injury and high yolk content of the egg make the cryopreservation of fish eggs and embryos more complicated than the freezing of sperm (Harvey and Ashwood-Smith 1982, Arii et al. 1992, Ujihira et al. 1994, Suzuki et al. 1995).

Variations in experimental designs are numerous, but surveys of results provide several overall impressions. A variation in cell permeability between non-activated and activated eggs has been demonstrated in northern pike *Esox lucius* and Atlantic salmon *Salmo salar*. Fertilized northern pike eggs incubated in buffered water with 10% DMSO were less permeable to DMSO than were unfertilized eggs incubated in 10% DMSO (Schmel and Graham 1986). Higher permeability coefficient for water through the perivitelline membrane in non-activated Atlantic salmon eggs was found compared to activated eggs (Loeffler and Lovtrup 1970). Inseminated and non-activated eggs have been used in cryopreservation of salmonid eggs (Zell 1978, Erdahl and Graham 1980, Stoss and Donaldson 1983). Variations in cell permeability during embryonic development also have been reported in zebrafish *Brachydanio rerio* in which water permeability of embryos increased several-fold between early blastula and the one-half epiboly stage (Harvey and Chamberlain 1982). Because in the half-epiboly stage, the embryo reaches a manageable size for cryopreservation (about 10 to 15 μm) and the blastodisc is thick enough for cryoprotectant to diffuse, it was suggested that the gastrulating embryo was the logical candidate for freezing experiments. Embryos at gastrula stage were chosen for freezing studies in Japanese medaka *Oryzias latipes* (Arii et al. 1987). Fish embryos underwent a change in tolerance or sensitivity toward low temperatures (chilling) (Dinnyes et al. 1997, Urbanyi et al. 1998, Zhang and Rawson 1995), cryoprotectants (Adam et al. 1995, Suzuki et al. 1995, Rana et al. 1995, Urbanyi et al. 1997) and mechanical disturbances (Gwo et al. 1995) during embryonic development. Generally, late embryo stages were more resistant to chilling injury, cryoprotectant toxicity and mechanical shock than were earlier embryonic stages (Sasaki et al. 1988, Gwo et al. 1995, Magnus et al. 1995, Baranyai et al. 1997, Urbanyi et al. 1997, 1998). However, the temperature tolerance of each embryonic stage seems to vary with fish species (Gwo et al. 1995) and different stages have been studied. The stages between the blastodisc (128 cells) and epiboly were chosen in attempts to freeze embryos of the Atlantic herring *Clupea harengus* (Whittingham and Rosenthal 1978); the heart formation stage in goldfish *Carassius auratus* (Liu et al. 1993), the heart-beat stage in zebrafish (Zhang et al.1993), the tail-bud stage (i.e. after closure of blastopore) in common carp *Cyprinus carpio* (Zhang et al. 1989) and eyed stage in rainbow trout *Oncorhynchus mykiss* (Haga 1982) were used in other freezing experiments. Even hatched Japanese medaka fry were used for freezing experiments (Onizuka et al. 1984, Onizuka and Egami 1985). The tolerance patterns of chilling and mechanical shock of fish embryos have been found to differ greatly. For example, two different ontogenetic tolerance patterns for mechanical and chilling shock have been shown for embryos of red drum *Sciaenopus ocellatus* (Gwo et al. 1995).

Dimethyl sulfoxide, methanol, EG and GLY are commonly used cryoprotectants at concentrations of between 0.5 and 2.0 M in artificial sea water (Whittingham and Rosenthal 1978), fish Ringer's solution (Harvey and Ashwood-Smith 1982, Onizuka et al. 1984, Onizuka and Egami 1985), artificial ovarian fluid (Stoss and Donaldson 1983), artificial seminal plasma (Erdahl and Graham 1980), isotonic electrolyte solution (3.2 mM KCl, 2.3 mM $CaCl_2 \bullet 2H_20$, 180 mM NaCl) (Haga 1982) and modified Hanks' solution (Harvey 1983a) for fish eggs and embryo freezing studies. Based on pre-freezing experiments, 1 M DMSO was chosen for rainbow trout (Stoss and Donaldson 1983), and herring (Whittingham and Rosenthal 1978). Methanol was more effective than either DMSO or EG for zebrafish embryo cryopreservation (Zhang et al.

1993). Although GLY penetrated into zebrafish embryos more efficiently than DMSO, the difficulty of removing it by dilution was a serious drawback (Harvey et al. 1983). It was also noted that 1 M DMSO did not affect the percent hatch of zebrafish exposed in the half-epiboly stage for as long as 1 hr at 23 °C, while 1 M GLY was toxic (Harvey et al. 1983). Herring embryos (4- to 8-cell stages and half-epiboly) withstood 2 hr in 1.5 M DMSO at 5 °C, while GLY and EG were toxic at the same molarity for the same periods (Whittingham and Rosenthal 1978). After equilibrium for 20 min at 23 to 25 °C, the maximum concentration tolerated by morulae and tail-bud stages of red drum were 1 M GLY, 2 M DMSO, 2 M methanol and 0.5 M sucrose; EG was toxic at the morulae stage even at 0.25 M, while the tail-bud stage showed resistance to 2 M (Robertson et al. 1988). Japanese medaka embryos were exposed to 1.5 M DMSO for 30 min at room temperature before they were frozen (Onizuka et al. 1984). The maximum nontoxic concentrations of cryoprotectants for the heart-beat stage in zebrafish embryos were 2 M for methanol, EG and DMSO, 1 M for GLY and 0.5 for sucrose (Zhang et al. 1993). Higher DMSO concentrations (1.5 to 2.0 M) increased the survival rate of herring and salmon embryos at temperatures of -7 to -10 °C (Whittingham and Rosenthal 1978, Erdahl and Graham 1980, Haga 1982). These data are consistent with results in zebrafish embryos in which higher concentrations of cryoprotectant (3 M) and a longer equilibration time (2 hr) at subzero temperatures generally increased embryo survival although the toxicity at this concentration was detrimental to hatching (Zhang et al. 1993).

Cryoprotectants, although essential for minimizing injury during freezing, are harmful to biological systems even at relatively low concentrations. The reduction of lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) enzyme activity were suggested to be due to the damage to the perivitelline membrane and blastoderm caused by the osmotic stress and denaturation of the leached enzymes by the cryoprotectant (Adam et al. 1995). A stepwise equilibration of non-activated eggs of coho salmon *Oncorhynchus kisutch* to 1 M DMSO for 10 min at 5 °C yielded better hatching than did a direct plunge (Stoss and Donaldson 1983). However, it was suggested that serial dosing was unnecessary, because there was no improvement with serial addition of cryoprotectants in morulae and tail-bud stages of red drum (Roberston et al. 1988). It was also reported that gradual stepwise addition of cryoprotectant did not reduce toxicity (Zhang et al. 1993), and reduction in temperature, from 25 °C to 0 °C, did not reduce the toxicity of cryoprotectants (Adam et al. 1995).

Zell (1978) and Erdahl and Graham (1980) claimed that rainbow trout eggs protected with 8 to 14% DMSO and frozen to -20 °C were viable after thawing. Harvey and Ashwood-Smith (1982) demonstrated that with the freezing rate (5 °C per min or more) used by the above authors, the surviving rainbow trout eggs were supercooled rather than frozen. Several workers have shown that slow freezing rates result in better survival of fish embryos after freezing. It was suggested the optimum freezing rate for salmonid eggs should be slower than 0.3 °C per min (Stoss and Donaldson 1983). A slow freezing rate (0.3 °C per min) was optimum for the heart-beat stage of zebrafish embryos (Zhang et al. 1993). Freezing rates of between 0.2 and 0.4 °C per min were commonly used in fish embryo experiments (Whittingham and Rosenthal 1978, Harvey et al. 1983, Onizuka et al. 1984, Liu et al. 1993, Zhang et al. 1993). Some success was obtained with freezing rates of less than 0.07 °C per min for the cryopreservation of common carp embryos (Zhang et al. 1989). Because even at freezing rates as low as 0.01 °C per min intracellular freezing is inevitable (Harvey and Ashwood-Smith 1982), and ice formation within the eggs may be the main factor limiting in the survival of fish embryos.

The survival rate of fish embryos declined abruptly below -20 °C. Viable herring embryos could be recovered from -20 °C, but only abnormal fry were obtained (Whittingham and Rosenthal 1978). With a freezing rate of -0.3 °C per min, Japanese medaka embryos cooled to -20 °C survived and developed to normal fry, but no viable embryos were observed with cooling to -30 °C (Onizuka et al. 1984). No zebrafish embryos hatched after cooling to -30 °C (Zhang et al. 1993). The hatching success of goldfish embryos at -35 °C was 1% (Liu et al. 1993). Inseminated salmonid eggs could withstand freezing to -55 °C (Zell 1978), but this could not be repeated (Erdahl and Graham 1980). Common carp embryos at tail-bud stage were frozen to -196 °C and yielded 25% survival after thawing, and 18% hatching (Zhang et al. 1989). A procedure was described that allowed survival of heart-beat stage embryos of loach *Misqurrus anguillicaudatus* by rapid freezing (Zhang et al. 1997). The embryos were first exposed at 4 °C to a solution containing 0.25 M DMSO, 0.2 M methanol (MeOH) and 0.01 M GLY in buffer MW-4 for 1 hr. The embryos were cooled at -2 °C per min from 4 °C to -20 °C, at -10 °C per min to -60 °C, and at -20 °C per min to -196 °C. With this method, 1 of 16 loach embryos was claimed to have hatched. Ultrastructural findings also demonstrated that little damage was induced with this rapid freezing and rapid thawing (40 °C) method.

Vitrification

Eggs and embryos are prone to injury from chilling, but only embryos past a specific developmental stage have the potential to survive cold exposure (Gwo et al. 1995, Hagedorn et al. 1997a, 1997b, Gwo and Lin 1998). Freezing of zebrafish embryos by controlled slow cooling methods showed that intra-embryonic ice formation was inevitable even when embryos were cooled with a high concentration of cryoprotectant (Zhang et al. 1993). Chilling injury and intracellular freezing may be prevented by vitrification using ultrarapid cooling. A complex solution consisting of DMSO, A, PG and polyethylene glycerol was devised for mammalian embryo freezing (Rall 1987). This solution vitrified, or in other words, formed a glassy solid at low temperatures without the formation of ice crystals. Vitrification could preclude the kinetic processes associated with chilling injury and therefore would be preferable for chill-sensitive embryos, like *Drosophila* (Rall 1993). Zebrafish frozen as 6-somite stage embryos by the vitrification method using a solution that consisted of butane-2, 3-diol, PG, and polyethylene glycol yielded 32% morphologically intact embryos immediately after vitrification, but failed to yield any survival after thawing (Zhang and Rawson 1996a). Embryos become temporarily opaque during warming, which was an indication of intraembryonic ice formation, especially following cracking of the glass. It was concluded that insufficient dehydration and permeation of cryoprotectants caused intraembryonic ice formation. It has been reported that the yolk syncytial layer (YSL), a vitelline membrane formed by a single cell that surrounds the yolk, and not the yolk or chorion (zona radiata) of zebrafish embryos blocked the permeation of cryoprotectants into the yolk (Zhang and Rawson 1996a, 1996b, Hagedorn et al. 1996, 1997a, 1997b). An understanding of the physiology and removal of this permeability barrier could be a key to the future successful cryopreservation of zebrafish embryos.

Cryopreservation of Penaeid Shrimp Eggs and Embryos

Penaeid shrimp eggs exhibit changes in sensitivity to mechanical shock at various stages of embryonic development (Robertson 1986, Gwo and Lin 1998). Handling procedures are not well tolerated by *Penaeus japonicus* embryos prior to about the 16-cell stage, about 2 hr after

spawning (Gwo and Lin 1998). Older embryos of *P. vammamei, P. monodon* (McLellan et al. 1992, Arun and Subramoniam 1997), *P. japonicus* (Gwo and Lin 1998), *P. indicus* (Newton and Subramoniam 1996), *P. aztecus, P. setiferus* and *P. stylirostris* (Robertson et al. 1988) were also relatively more resistant to higher cryoprotectant concentrations and longer equilibration times than were younger embryos. The increase in resistance to cryoprotectants observed with shrimp embryonic development may be related to the progressive increase in the osmotic regulation and membrane permeability of the embryo. Embryos acquire structural organization about 5 hr after fertilization, and the establishment of osmotic regulation is considered possible at this time (Simon et al. 1994).

Egg activation in penaeids initiates a series of cortical changes that coincide with the loss of the vitelline envelope and the formation of the hatching (fertilization) envelope. The hatching envelope, which contains proteins and carbohydrates, consists of an outer electron-dense layer and an inner translucent layer (Pillai and Clark 1988, 1990). The hatching envelope becomes a barrier for various compounds including cryoprotectants (Pillai and Clark 1988, 1990, Lynn et al. 1991, Simon et al.1994, Clark et al. 1996). Water could easily penetrate the hatching envelope of the Chinese prawn *P. orientalis*, but DMSO and GLY could not (Zhang et al. 1992a, 1992b). The hatching envelope is a protective extracellular matrix that remains with the developing embryos through formation of the nauplius larva. This envelope seems to become more permeable as the embryo ages, probably reflecting the aging of its structure when in contact with seawater (Simon et al. 1994). For example, in *P. indicus* the hatching envelope did not behave as a barrier for methanol in embryos 5 hr after fertilization (Simon et al. 1994). On the contrary, cryoprotectant permeation through the hatching envelope of *P. indicus* was observed as early as the morula stage (Newton and Subramoniam 1996).

The cryoprotectant ability of methanol was better than the other cryoprotectants (GLY, DMSO, EG and PG) tested in five developmental stages (morula, blastocele, pre-nauplius, sec nauplius and first zoea) of *P. japonicus* (Gwo and Lin 1998). The poor qualities of GLY as a cryoprotectant of *P. japonicus* embryos (Gwo and Lin 1998) had also been shown earlier in the Pacific oyster (Gwo et al. 1995), rotifer (Bedding et al. 1987) and sea urchin (Asahina and Takahashi 1978, 1979). These results are in agreement with data reported for embryos of *P. indicus* (Simon et al. 1994, Newton and Subramoniam 1996) and *P. monodon* (Arun and Subramoniam 1997). Dimethyl sulfoxide has proven to be toxic to *P. japonicus* and *P. monodon* embryos, nauplii and zoea (Arun and Subramoniam 1997, Gwo and Lin 1998). Exposure to DMSO could also result in deformities in sea urchin embryos (Asahina and Takahashi 1979). The toxicity of DMSO could be reduced or neutralized when DMSO fractions were increasingly replaced by EG. The use of dual cryoprotectants to reduce the toxicity of DMSO was demonstrated in *P. monodon* embryos (Arun and Subramoniam 1997). The variation in the level of toxicity of these cryoprotectants may reflect differences in molecular weights and interactions with the biological samples. Methanol has a low osmolality and 10% (2.5 M) methanol extended with 35 ppt sea water has an osmolality equivalent to about 1,000 mOsmol/Kg. Methanol quickly penetrates through the plasma membrane without affecting embryo and larval survival (Asahina and Takahashi 1979, Zhang and Rawson 1995, Newton and Subramoniam 1996, Arun and Subramoniam 1997). Permeation of other cryoprotectant compounds (e.g. GLY and PG) is slow and appears to be negatively correlated with molecular weights. Rates of cryoprotectant permeation are also related to embryonic stages and are species dependent. Dimethyl sulfoxide penetrated only gastrula, while smaller molecules (methanol and EG) entered embryos 5 hr after fertilization in *P. indicus* (Simon et al. 1994). Addition of DMSO caused re-expansion of the *P.*

monodon nauplius and zoea within 30 sec (Arun and Subramoniam 1997). After comparison with mammalian embryos, which are relatively easily permeated and have low toxicity thresholds to cryoprotectants (both duration and concentration), it has been suggested that the toxicity of cryoprotectants could be of osmotic rather than of biochemical origin in shrimp embryos (Simon et al. 1994). However, GLY could not completely penetrate into the nauplii of *P. monodon* and caused a late toxic reaction which may be primarily biochemical rather than osmotic (Arun and Subramoniam 1997).

Methanol has been reported to have superior cryoprotective properties for a variety of cell types including fish sperm (Harvey 1983b, Rana and McAndrew 1989, Lahnsteiner et al. 1996, Gwo et al. 1999), red drum embryos (Robertson et al. 1988) and *schistosoma* larvae (James 1980). The toxicity of this compound is dependent on two main factors: temperature and time. Damage is reduced at lower temperatures and with shorter exposure times. The cryoprotective effects of methanol and a mixture of methanol with slowly penetrating cryoprotectants (DMSO, EG or PG) were compared in *P. indicus* embryos at 5 hr after fertilization and at gastrula with no significant difference in survival (Simon et al. 1994). However, higher survival of *P. semisulcatus* was found when a mixture of GLY and DMSO was used rather than GLY alone (Diwan and Kandasami 1997). The addition of sugar (sucrose) to GLY or methanol showed beneficial effects for *Artemia salina* (Baust and Lawrence 1977) and zebrafish embryos (Zhang and Rawson 1995). Sucrose, a non-reducing disaccharide of glucose, has been found to maintain membrane integrity during dehydration (Crowe and Crowe 1984). Sugars dehydrated the zebrafish embryos before and during freezing, which served to reduce intracellular ice crystal formation (Zhang and Rawson 1995). Sucrose had no toxic effects but at higher concentrations exhibited osmotic effects (Arun and Subramoniam 1997). Exposure of the nauplii and zoea of *P. monodon* to high concentrations of sucrose caused rupture and oozing of the body contents and resulted in total mortality (Arun and Subramoniam 1997).

Chilling tolerance is age dependent. The sensitivity of shrimp embryos to chilling injury depends upon the developmental stage and the cryoprotectant used. The earlier the developmental stage, the more rapidly *P. japonicus* embryos died when held at 0 °C (Gwo and Lin 1998), and embryo survival decreased as the duration of exposure to low temperature was increased. The larvae (nauplii and zoea) of *P. monodon* were intolerant to supercooled (-4 °C) temperatures (Arun and Subramoniam 1997). Studies involving various animal embryos suggest that a high sensitivity to chilling injury is associated with large amounts of intraembryonic lipids and yolk granules (Polge and Willadsen 1978, Nagashima et al. 1995, Zhang and Rawson 1995).

Later developmental stages have been found to be less sensitive to increases in cryoprotectant concentration than are earlier stages. The zoea stages of *P. monodon* showed higher tolerance to cryoprotectants and osmotic shock than did nauplii (Arun and Subramoniam 1997). Only larval stages of *P. japonicus* survived after freezing to -20 °C with slow cooling rates (-1 and -5 °C per min) in 10% methanol (Gwo and Lin 1998). Survival of 82% was reported for *P. indicus* nauplii cooled to -30 °C and 63% at -40 °C with a slow cooling (-1.5 °C per min) method in 20% EG (Subramoniam and Newton 1993). With the use of a faster freezing rate (-30 °C per min), the viability of *P. monodon* larvae was extended beyond -40 to -70 °C (Arun and Subramoniam 1997). The presence of supercooled fluids and ice formation may each be critical factor in the survival of shrimp larvae. Cooling rates of between -15 to -30 °C per min to final temperatures of -50 to -70 °C yielded 85% to 95% survival of *P. monodon* larvae (Arun and Subramoniam 1997). Despite some larvae that demonstrated twitching movements and weak

motility when thawed from -196 °C, metamorphosis was not achieved (Gwo and Lin 1998) and no viable larvae were obtained.

Cryobiology of Eggs and Embryos

Attempts to develop cryopreservation procedures for eggs and embryos of aquatic organisms have been based primarily on empirical approaches. Mammalian-type freezing regimens were not suitable for cryopreservation of fish and shrimp embryos (Robertson et al. 1988, Gwo et al. 1995, Zhang and Rawson 1996a, 1996b, Arun and Subramoniam 1997). Considerable modification is required to achieve desired results. Information on biophysical parameters of eggs and embryos from aquatic organisms required to predict optimal freezing protocols has begun to accumulate. These parameters include plasma membrane permeability to water (L_p; water permeability; hydraulic conductivity; phenomenological water permeability) and cryoprotectants (ω), the temperature-dependence of these permeabilities and the probability of intracellular ice formation (IIF) and median IIF temperature as a function of cooling rate and the suspending medium.

The large size and sphericity of the eggs of aquatic organisms makes them amenable to osmometric and cryobiologic studies. The volumetric response to anisotonic conditions has been studied in eggs from several species, including the small abalone *Haliotis diversicolor* (Lin 1992) and hard clam *Meretrix lusoria* (Lin et al. 1993b). The plot of equilibrium egg volume versus 1/osmolality of the suspending medium was linear for each species examined, indicating that the eggs behaved as ideal osmometers. Extrapolating this relationship to infinite osmolalities gives an estimate of the fractional volume of the egg that is occupied by non-osmotically active solids (FV_b; osmotically inactive volume). Estimates of FV_b for eggs of hard clam and small abalone ranged from 0.47 to 0.54 which were higher than that of mammalian oocytes (0.1 to 0.2; Lin 1992, Lin et al. 1993a, 1993b). The FV_b value of 0.80 for gastrula stage zebrafish embryos is the highest reported for eggs or embryos (Zhang and Rawson 1996b), and was attributed to the large amount of yolk.

With knowledge the value of FV_b, one can estimate the L_p of eggs and embryos by use of cell volume measurements collected as a function of elapsed time during osmotic equilibration. A mean L_p value of 6.3×10^{-9} m/s-atm has been reported for eggs of small abalone at room temperature (Lin 1992). A higher mean L_p value of 23.1×10^{-9} m/s-atm was measured for eggs of hard clam (Lin et al. 1993a, 1993b). The estimated mean L_p for zebrafish embryos (0.022 to 0.1 μm per min-atm) was less than those described for *Drosophila* and mice (Hagedorn et al. 1997b). The low L_p indicated that cellular water moved out of the embryo slowly. The effect of temperature on permeability is different for different solutes, for different developmental stages, and for the same stage of different species. For example, the L_p changed during the development of zebrafish (Hagedorn et al. 1997b). Because L_p is temperature-dependent, a quantitative understanding of this dependence below 0 °C is needed to improve the accuracy of the predictions of thermodynamic models.

Quantitative information on cryoprotectant permeability (ω) of eggs and embryos is limited. For PG, the ω value of 0.05 μm per sec for zebrafish embryos was smaller than that reported for mammalian eggs, which ranged from 0.2 to 0.82 μm per sec (Zhang and Rawson 1996b). Based on visual estimates of shrinkage and swelling and the time required to return to the original isotonic volume, it was concluded that the order of permeability of cryoprotectants for eggs of small abalone was PG followed by DMSO, A, EG and GLY (Lin 1992). Small

abalone eggs were more permeable to PG, DMSO, A and EG (10 to 20 min to equilibrate at 20 °C) than they were to GLY (impermeable). Similar results were also reported with eggs of the hard clam (Lin et al. 1993b).

The probability of IIF increased with time and lower temperatures. The median IIF temperature in salt solutions has been assessed to be -22.5 for eggs of the Pacific oyster and -21.0 °C for embryos (Lin and Lung 1995). The transition temperature zone of IIF probability from 0 to 1 was broader for embryos than for eggs. Currently, estimates of the most critical biophysical parameters are available for few species. Information on shrimp eggs and embryos necessary for the prediction of improved cryopreservation protocols is lacking. Certain parameters with similar values among the species examined to date, such as mean IIF temperature, may require less rigorous study across species. However, accurate estimates of L_p and other parameters will be essential for developing models to predict optimal cryopreservation procedures.

Future Prospects

Diffusion barriers prevent cryoprotectant equilibration and osmotic dehydration in large cell assemblages. Using a standard two-step cryobiological procedure, it was demonstrated that 84% of isolated intact single cells from zebrafish blastoderm embryos survived freezing to -196 °C (Harvey 1983a). An abrupt drop (26%) in survival was observed when clumps of 5 to 10 embryonic cells were frozen with the same procedures. For intact blastoderm the proportion of viable cells was reduced to 2%. It was concluded that insufficient cryoprotectant penetrated to inner cells and the inability of water to exit through outer layers during cooling caused the death of the embryonic cells. Study of 6-somite stage zebrafish yielded 32% morphologically intact embryos immediately after vitrification although none survived after thawing (Zhang and Rawson 1996b). It was also reported the insufficient dehydration and permeation of cryoprotectants caused intraembryonic ice formation. The YSL, not the yolk or chorion, of fish embryos blocks the permeation of cryoprotectants into the yolk (Yu et al. 1991, Zhang and Rawson 1996a, 1996b, Hagedorn et al. 1997a, 1997b, Liu et al. 1997). The understanding of the physiology of this permeability barrier will be a key to the future successful cryopreservation of fish embryos. Exposure of fish eggs to cryoprotectants in a vacuum might attain successful penetration of cryoprotectant and subsequent cryopreservation. In a preliminary trial, a two-step freezing to -196 °C in a DMSO-based cryoprotectant solution, following equilibration under vacuum, yielded fertilization of eggs of Fire-tailed Gudgeon *Hypseleotris galii* and Australian bass *Macquaria novemaculeata* (Leung and Jamieson 1991).

Egg activation in penaeid shrimp initiates a series of cortical changes that coincide with the loss of the vitelline envelope and the formation of the jelly layer, surface coat and hatching envelope. Early-stage shrimp embryos have a complex membrane system comprising an outer hatching envelope and an inner ooplasma. The external impervious chitinous body wall and a layer of basement membrane with glands cover the body mass of late-stage larvae (nauplii and zoea). None of the cryoprotectant solutions tested (DMSO, EG, PG, GLY and methanol) appeared to permeate this membrane system. To obtain survival of cryopreserved shrimp embryos, it will require developments in the techniques of membrane permeabilization rather than simply changes in cryopreservation methods. A hypotonic loading technique was used to increase the permeation of cryoprotectant into the nectochaete larvae of ragworm *Nereis virens* (Grout et al. 1992). Larvae were transferred into a hypotonic solution for a short period of time

and were immersed in a hypertonic mixture, yielding 35 to 48% larval survival after cryopreservation. Uses of combinations of cryoprotectants (penetrating and non-penetrating) and cold acclimation before freezing may protect late-stage penaeid shrimp embryos during freezing (Lawrence and Baust 1980, Grout et al. 1992, Arun and Subramoniam 1997, Gwo and Lin 1998). Larvae of *P. monodon* could be frozen to a lower temperature by improving the cryoprotectant permeation and dehydration procedures, prior to storage under LN_2 (Arun and Subramoniam 1997). Slow cooling rate (< -1 °C per min) caused pre-freeze mortality of shrimp larvae due to supercooling of the cytoplasm or the external cryoprotectant (Arun and Subramoniam 1997). Nauplii of *P. indicus* survived concentrations of EG and methanol as high as 12 M (Subramoniam and Newton 1993). Use of a mixture EG and DMSO (25:15, V/V) yielded 90% survival of *P. monodon* zoea after 60 min of equilibration at 27 °C (Arun and Subramoniam 1997).

The possibility of using vitrification to cryopreserve penaeid shrimp larvae in the future is warranted. The approaches used to overcome obstacles to cryopreserving large insect embryos such as those of the fruit fly *Drosophila melangogaster* and malaria mosquito *Anopheles gambiae* may provide insights useful for cryopreservation of early-stage shrimp embryos (Mazur et al. 1992, Steponkus and Caldwell 1993, Valencia et al. 1996). It has been suggested that cell differentiation and diversity are not the barriers to successful cryopreservation of embryos of aquatic organisms, but rather it is the specific cell organization associated with early embryonic formation and large cell size are the problems (Grout et al. 1992). The information on shrimp eggs and embryos necessary for prediction of improved cryopreservation protocols is lacking. Accurate estimates of L_p and other parameters will be essential for developing models to predict optimal procedures for cryopreservation of eggs and embryos.

Cryopreservation of sperm is successful, but because there are no successful protocols for the cryopreservation of fish eggs and embryos**,** the mitochondrial DNA inherited maternally through the egg cannot be preserved at present. However, individual blastomeres have survived freezing and thawing using cells isolated from blastoderm of zebrafish, rainbow trout and African catfish *Clarias gariepinus* (Harvey 1983a, Nilsson and Cloud 1993, Magyary et al. 1994, Leveroni Calvi and Maisse 1997). Isolated embryonic cells from rainbow trout and zebrafish have been successfully transplanted into recipient blastulae (Nilsson and Cloud 1993, Lin et al. 1992). The pluripotent donor cells participated in the development of the recipient embryo and also contributed to the germ line of the resultant chimeras (Lin et al. 1992, Magyary et al. 1994). A proportion of these transplanted cells would be expected to enter the germ line and subsequently develop into eggs (Nilsson and Cloud 1993). Nuclei from the cells of blastula embryos can be transferred into enucleated eggs and give rise to diploid individuals with the nuclear genome of the transplanted nucleus (Tung 1980). Freezing of individual egg nuclei or blastomeres and reestablishment of these after thawing into the germ line of recipient embryos or a combination of nuclear transplantation may be alternative approaches to preserve the complete diploid genome of fishes.

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Summary of Recent Developments and Advances by Author

An increasing number of wild fish species are in danger of extinction, often as a result of human activities. Gametes (sperm and egg) and embryo cryopreservation are important tools for the development of *ex situ* conservation of genetic diversity and restoring threatened species of animal species (Thorgaard and Cloud 1993). The conservation of both paternal and maternal genetic information is essential; however, cryopreservation of fish eggs and embryos has been relatively unsuccessful. Cryopreservation of fish semen has been successful for many teleost species (Lubzens et al. 1996, Maisse 1996, Gwo 1999, 2000a,b). The combination of sperm cryopreservation with androgenesis offers a way of regenerating a stock using only the sperm as a source of nuclear genomic material (Araki et al. 1995, Nagoya et al. 1996). Unfortunately, this technique does not overcome the loss of mitochondrial DNA, which is inherited maternally (Komen and Thorgaard 2007). Successful cryopreservation of intact fish embryos has not yet been accomplished (Gwo 2000b).

One way to maintain the genetic diversity of both nuclear genome and mitochondrial DNA is the cryopreservation of isolated blastomeres (Table 1) that could be transplanted into host (recipient) blastulae after thawing (Harvey 1983, Lin et al. 1992, Nilsson and Cloud 1992, 1993, Calvi and Maisse 1998, Stüssmann et. al.1999, Kusuda et al. 2002). Because primordial germ cells (PGC) are the progenitor of the germ cell lineage and is committed to differentiate into either spermatogonia or oogonia after the completion of gonadal differentiation, it has the potential to develop into a complete individual (Yoshizaki et al. 2002, Saito et. al. 2008). Therefore, preserving PGC is an alternate novel way to preserve the fish genetic resources (Kobayashi et al. 2003, 2004, Takeuchi et al. 2003, 2004). PGC cryopreservation, in combination with PGC xenotransplantation, would be a useful strategy for gene banking and helping the conservation of endangered species (Takeuchi et al. 2004).

Table 1. Cryopreservation of blastomeres from some finfish species

Fish species	*Oncorhychus mykiss*		*O. keta*	*Brachydanio rerio*	*Carassius auratus*	*Oryzias latipes*	*Odontesthes bonariensis*	*Sillago japonica*
Common name	rainbow trout		Chum salmon	zebrafish	goldfish	medaka	pejerrey	Whiting
Method	straw-PF cryobival	starw-PF	straw-PF[b]	vial-alcohol bath	starw-PF	Straw dry ice/ethanol	Straw dry ice/ethanol	straw dry ice/ethanol
Volume(μl)	500; 1800	250	250	5000	250	250	250	250
Cryoprotectant	8.7% DMSO	1.4M 1,2-propanediol	10% DMSO, 10%FBS	1M DMSO	10% DMSO, 10%FBS[f]	9% DMSO, 10%FBS	18% DMSO, 10%FBS	9% DMSO, 10%FBS
Extender	Ca^{2+}-Mg^{2+} free Niu Twiffy's soln.	Ca^{2+}-Mg^{2+} free dissociating soln.[d]	MEM[c]	medium 199[e]	MEM	culture medium[g]	culture medium	culture medium
Equilibration time(min)	10	25	30	30	30			
Cooling rate(℃/min)	0.3-2	0.3-2	1		1	1	1	14
Seeding temp.(℃)	-4 or -7	-6.6	-7	-6	-7	No seeding	No seeding	No seeding
Temp.(℃) before plunged into LN_2	-80 or -90	-80 (10 min)	-30	-25	-30	-60	-60	-60
Preserved duration	48h		>3min		54-60 d	1.5h	1.5h	1.5h
Thawing Temp.(℃)	0	0; melting ice	15 water bath	room temp.	15 water bath	20 water bath	20 water bath	20
Thawing Time(s)	300	600	20		20	60	60	60
Thawing Rate(℃/min)		20		43				
Embryonic stage	Blastula (Isolated blastomeres)	blastula	blastula	gastrula	blastula	512-cell stage	52~1024-cell stage	256-cell stage
Post-thaw survival[a] (%)	Intact morphology; 16~39	Morphology, metabolic & mechanical activity 53~95	Trypan blue dye exclusion 59	85	44-55	34	67	20

[a]Post-thaw survival; absolute survival rate
[b]PF; programmable freezer
[c]MEM; Eagle's minimum essential medium
[d] Ca^{2+}-Mg^{2+} free dissociating solution; 106mM NaCl, 3mM KCl, 100mM sucrose, 3g/l BSA, 20mM $CaCl_2$, 5mM Heps sodium salt, 15mM Bicine (pH 7.5, 315 mOsm/kg)
[e]medium 199; medium 199(10 × concentrate, with Hanks'salts and L-glutamine)10ml, 0.25M Heps buffer 10ml, 5mg/ml Kanamycin sulfate 1ml, 1M Sodium pyruvate 0.1ml, 8.8% $NaHCO_3$ 1.0ml, Fetal calf serum 15 ml,Distilled water 77.9ml
[f]FBS;fetal bovine serum
[g]culture medium; Ca^{2+}-free, Hepes-buffered Waymouth's culture medium MB 752/1 (pH 7.4,276 mOsm/kg)

Kobayashi et al. (2007) and Gwo et al. (unpublished data) developed a protocol for the use of rainbow trout *Oncorhynchus mykiss* primordial germ cells (PGCs) and document the restoration of live rainbow trout fish from gametes derived from these cryopreserved progenitors. The whole genital ridge (GR), the embryonic gonad containing PGCs, was excised at 30-d-post-fertilization and left to cryopreserve in a basal medium. Cryoprotection was provided by addition of 10% (V/V) ethylene glycol. Samples were loaded into the cryotubes and slowly (-1 °C /min) frozen to -80 °C before being plunged into liquid nitrogen. Morphological evaluation was performed using microscopy and video recording. The survival rates of post-thaw PGCs were determined by their green fluorescent intensity and ability to exclude trypanblue dye. Statistical evaluation suggested that freezing tolerance of whole GR is better than that of dissociated PGCs. Very low (8.1 ± 6.9%) viable PGCs were recovered using uncontrolled cooling protocols directly plunged into -80°C freezer. The controlled cooling rate is more effective than those of uncontrolled. A simple two-step cryopreservation protocol proved to be suitable for rainbow trout GR. Survival rates of rainbow trout GR was best observed using a cooling rate of 1 °C/min to -80 °C and then plunged into liquid nitrogen with the addition of 10% ethylene glycol. The thawed PGCs that were transplanted into the peritoneal cavities of allogenic trout hatchlings differentiated into mature spermatozoa and eggs in the host gonads. Furthermore, the fertilization of eggs derived from cryopreserved PGCs using cryopreserved spermatozoa resulted in the development of fertile F1 fish.

As the number of PGCs was limited (< 100 per rainbow trout embryo), Okutsu et al. (2006) transplanted adult testicular germ cell (contains isolated spermatogonial stem-cells) of rainbow trout into newly hatched sexually undifferentiated allogeneic embryos. The donor spermatogonia stem-cells showed a developmental plasticity and sexual bipotency, some produced mature spermatozoa in male hosts and others differentiated into fully functional eggs in female hosts. Normal live offsprings were produced from the hosts. Spermatogonia, retained the self-renewal ability, easily obtaining numerous from male fish of any age and was small enough to freeze. Furthermore, this technique can produce rainbow trout eggs by transplanting cryopreserved spermatogonia into sterile xenogenic surrogate masu salmon *Oncorhynchus masou* hatchlings producing only donor-derived offspring (Okutsu et al. 2007). Both PGC and spermatogonia cryopreservation techniques represent promising tools in efforts to save threatened fish species and endangered salmonid populations (Figure 1). Moreover, this surrogate breeding approach has significant potential for maintaining domesticated fish strains carrying commercially valuable traits for aquaculture purposes. Using fish species with small eggs and larvae compared with salmonids, Saito et al. (2008) demonstrated that xenogenesis also is realistic and practical across species, genus, and family barriers and can be achieved by transplantation of a single PGC from a donor species. Mass seed production of bluefin tuna *Thunnus thynnus* or grouper *Epinephelus malabaricus* offsprings is possible, by transplanting their PGCs or spermatogonia into sterile mackerels *Scomber japonicus* or black sea breams *Acanthopagrus schlegeli* which are earlier to mature, smaller in body size, easier to manipulate in captivity, and cheaper to maintain in terms of time, cost, space and labor. Inbred strains with desirable genetic characteristics can be rapidly generated by self-fertilizing male and female hosts derived from the same donor.

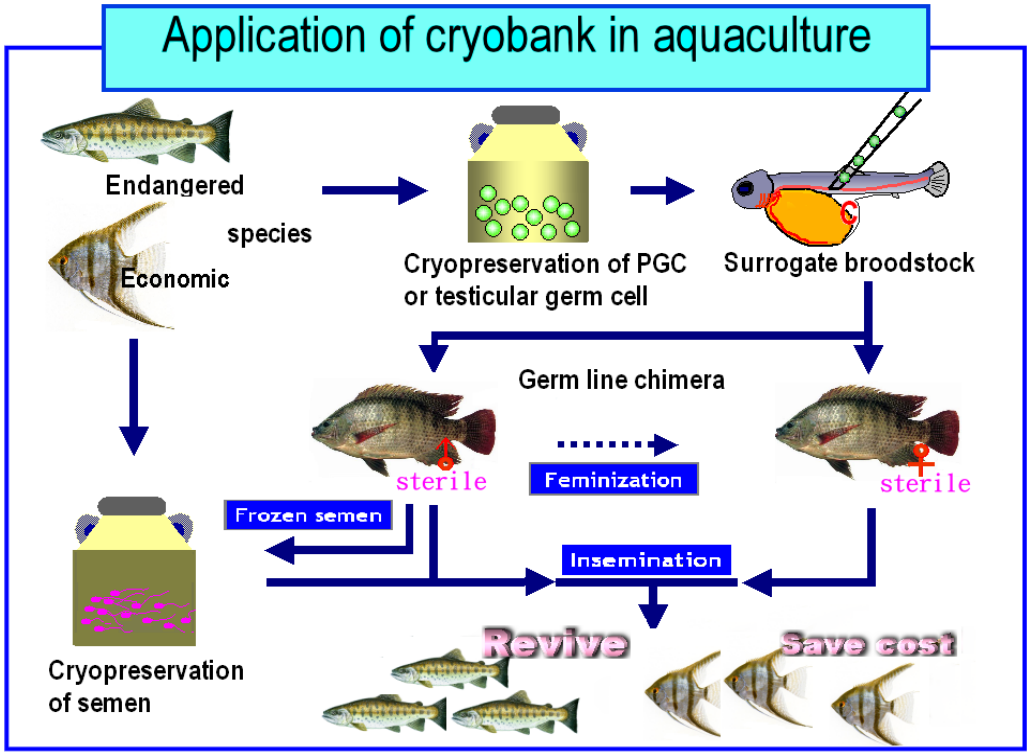

Figure 1. The creation of aquatic organism cryogenbanks is motived not only by the development of genetic breeding programs in aquaculture but also by conservation of biodiversity (genetic resources). *Ex situ* preservation (cryopreservation) could guarantee the enrichment and the restoration of endangered species due to climate change, pollution and excessive exploitation. Cryogenbanks can substantially extend generation lengths of small captive populations to retain higher levels of genetic diversity. Long-term storage and utilization of cryogenbanks could serve to mass produce economicly important species (grouper, tuna, ornamental aquarium fishes) with the added benefit of reduced labor, space and expense.

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VI. Cryopreservation of Gametes and Early Life Stages of Invertebrates

Cryopreservation of Molluscan Sperm: Pacific Oyster, Green-lipped Mussel, and Paua Abalone

Serean L. Adams, John F. Smith, H. Robin Tervit, Lindsay T. McGowan, Rodney D. Roberts, Achim R. Janke, Nick G. King, Samantha L. Gale and Stephen C. Webb

Most of global molluscan aquaculture production is based on wild-caught juveniles. The genetic makeup of those juveniles is beyond the control of the farmer, so stock performance will reflect the variability found in source populations. Terrestrial farming industries have domesticated stocks by controlling juvenile production and undertaking selective breeding. This approach has yielded huge gains over many decades. The more recent advent of selective breeding in aquaculture has already demonstrated gains with increases in growth rates of 9 to 17.5% (average 13%) per generation across a range of fish and shellfish species (Gjedrem 2002). The cryopreservation of sperm and eggs offers many advantages in selective breeding. The maintenance of live broodstock from numerous lines is expensive, and live animals can be lost through disease, algal blooms, storms or human error. The certainty of supply provided by cryopreservation allows exact planning of mating crosses, facilitating the implementation of breeding designs. The Cawthron Institute has developed larval rearing systems for oyster and mussel family production that allow ~95% of crosses to be successfully reared from D-stage larvae to spat (King et al. 2005), and selective breeding programs have been initiated in recent years (King et al. 2004, King and Janke 2006, King 2008). Selective breeding and reseeding programs are also underway for the New Zealand black-footed abalone, more commonly known by its traditional maori name, Paua (Adams 2007, Roberts personal communication).

The Cawthron Institute cryopreservation program is dedicated to providing practical systems for the cryopreservation of sperm, eggs and larvae from molluscan species to underpin breeding programs (Roberts et al. 2000a). This chapter outlines the approach and results relating to the cryopreservation of sperm from three molluscan species: Pacific oyster *Crassostrea gigas*, green-lipped mussel *Perna canaliculus* (also known as greenshell™ mussels), and Paua abalone *Haliotis iris*.

Collection and Handling of Gametes

The method of gamete collection and holding temperature markedly affects the interval that gametes from the various species remain viable for use in cryopreservation and fertilization applications.

Pacific Oyster

Sexually mature Pacific oysters were obtained from marine farms in New Zealand during their natural breeding season (November to December) or from conditioned broodstock. Sperm were obtained by "dry" stripping of the gonad (i.e., without addition of seawater) and stored on ice or at 5 ^{o}C. Eggs were strip-spawned, washed and resettled in ~30 mL of 1-µm filtered seawater (FSW) and stored at 5 ^{o}C until use (Adams et al. 2004, Tervit et al. 2005, Adams et al. 2008a). Sperm concentrations were determined prior to cryopreservation using a Neubauer

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haemocytometer. Egg density was determined by counting four 20-µL aliquots from a known dilution of concentrated eggs. The desired egg density was achieved by dilution with FSW.

In general, a large mature male will produce around 7 mL of dry sperm at a concentration of ~2 x 10^{10} cells/mL (Smith et al. 2001). When held "dry" at 5 ^{o}C, oyster sperm remained viable and suitable for cryopreservation for at least 5 d before a major decline in fertility occurred (Figure 1) (Roberts et al. 2004).

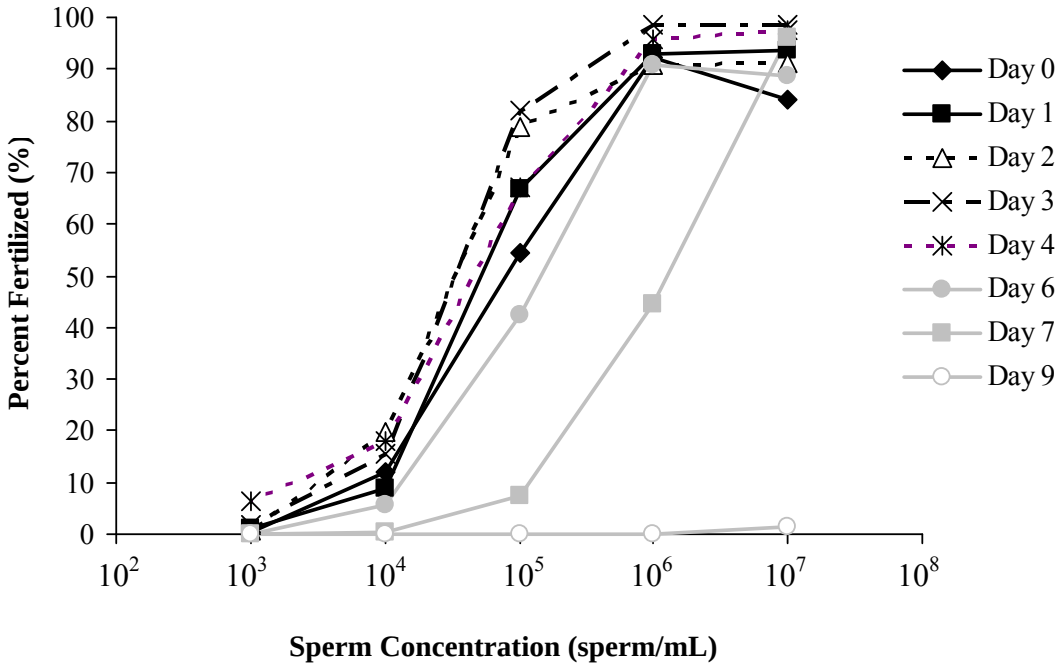

Figure 1. Effect of refrigerated storage on post-thaw fertility of Pacific oyster sperm. Data are from one pool of sperm from three males.

Green-lipped Mussel.

Thermal cycling with seawater between 18 and 10 ^{o}C was used to induce the mussels to spawn (Adams et al. 2009). Spawning mussels were removed when gamete emission was seen to be strong and sustained. At the onset of spawning, males were rinsed, placed anterior-up in 70-mL containers and left to spawn "dry". Concentrated sperm dripped from each male into the container and was collected every 30 min and stored at 5 ^{o}C until samples were pooled for use in experiments. Sperm concentration was determined as described above. Spawning females were rinsed in freshwater and placed in individual containers with ~500 mL of FSW at 10 ^{o}C. Females were allowed to spawn for 10-20 min and any eggs were discarded (to avoid use of already fertilized eggs), and the container was rinsed and refilled with clean seawater. Eggs were then collected every 30 min and each collection stored separately at 5 ^{o}C. A sample of eggs from each collection was examined for fertilization (indicated by the presence of polar bodies or cleavage approx 30 min after collection) before eggs were pooled. Egg density was determined as above.

Male mussels vary in the amount of sperm produced (range 0.5 to 10 mL of dry sperm at a concentration of ~5 x 10^9 cells/mL) with the majority of animals producing between 1 and 5 mL. When held "dry" at 5 ^{o}C, mussel sperm remained viable for cryopreservation for at least 4 d before a major decline in fertility occurred (Figure 2).

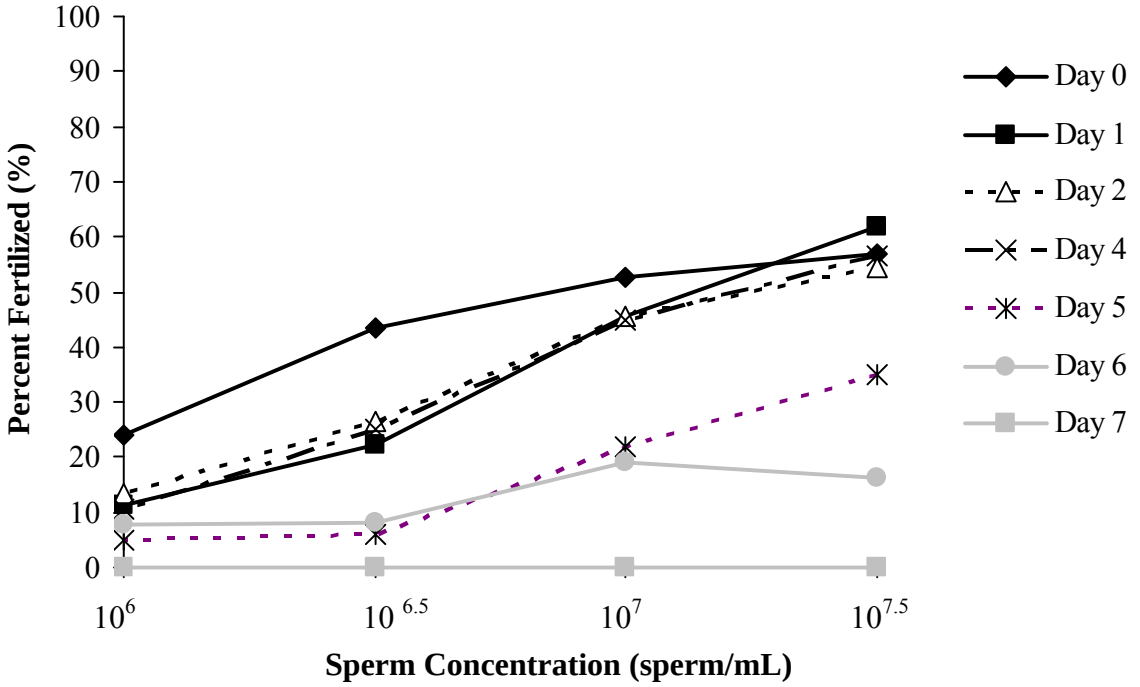

Figure 2. Effect of refrigerated storage on post-thaw fertility of mussel sperm. Data are from one pool of sperm from five males.

Paua Abalone

Abalone were spawned using chemical stimulation (Morse et al. 1977, Tong et al. 1992). Mature animals were sexed according to gonad color and immersed in FSW containing 250 ppm hydrogen peroxide and 1.3 mM NaOH and placed in the dark. Males and females were treated separately. After 3 hr, animals were washed thoroughly with FSW treated with ultraviolet light (UV). Animals were left in the dark in UV-treated seawater and checked every 30 min. Males were removed upon spawning and placed anterior downwards in separate containers without seawater. Sperm was collected into a separate container every 30 min and placed at 5 ^{o}C. Eggs from spawning females were siphoned into separate 20 L buckets and onto 80-µM mesh sieves. The eggs were concentrated into a 60-mL container and maintained at ~16 ^{o}C.

Abalone sperm in a "dry" state remained viable at 5 ^{o}C for about 3 d, but was usually used within 24 hr. Egg viability declined more rapidly so eggs were used for cryopreservation experiments within 3 hr of spawning.

Fertilization Assays

To evaluate the effectiveness of sperm cryopreservation techniques, a miniature sperm dose-response fertilization assay system was developed. This provided a direct measure of the required end point rather than indirect measures such as sperm motility and viability.

Pacific Oyster

The assay method for oysters has been described in detail (Smith et al. 2001, Adams et al. 2004, Adams et al. 2008a). Accordingly, the assays were carried out in 12-well tissue culture plates. Briefly, each well contained ~600 eggs in 3 mL of FSW. Sperm were added to each well to yield final concentrations ranging from 10^2 to 10^6 sperm/mL for fresh sperm, and from 10^5 to 3.2×10^7 sperm/mL for cryopreserved sperm. This represents a range of sperm-to-egg ratios of 0.5:1 (10^2/mL) to 160,000:1 (3.2×10^7/mL). Sperm fertilization assays were conducted in duplicate. Plates were incubated at 21 ± 2 °C for 2.5 to 3 hr until embryos had reached at least the 4-cell stage. Formalin was added (~0.5% final concentration) to arrest further development and the percentage of eggs fertilized was determined by counting ~100 eggs and embryos per well. Occasionally, eggs with polar bodies but no cleavage were observed and were counted as unfertilized. The concentration of sperm required to achieve 50% fertilization (EC50) was calculated using a software application "Flexi 3.04" that involves a mixed-model smoother and a logit link function to fit curves to the fertilization assay data (Upsdell 1994).

Statistical comparisons of fertility compared EC50 values using the Minitab Software (Minitab Inc., State College, PA, USA). Adoption of changes to cryopreservation protocols were based on treatments giving a significantly lower sperm concentration to achieve EC50.

Green-lipped Mussel

A fertility assay system similar to oysters was developed for mussel sperm with the major differences being the need to have the sperm and eggs in FSW containing 0.1% bovine serum albumin (Albumax I Lipid Rich BSA, Gibco™ Invitrogen Corporation, New Zealand) as a surfactant to prevent sperm adhesion. The incubation temperature was reduced to 18 ^{o}C.

Paua Abalone

For abalone, a plate assay similar to that for oysters was developed (Roberts et al. 2000b) but with only 50 eggs/mL. The assay was maintained at 16-18 ^{o}C.

Larval Development Assays

Cryopreservation protocols were developed to optimize fertilization levels in the fertilization assays. These protocols were subjected to a larval development test to ensure the developmental competence of the fertilized embryos.

Pacific Oyster

Larval D-stage yield for oysters was quantified as follows: One million eggs were fertilized in 10 mL of FSW at a ratio of 2,000 cryopreserved sperm/egg. After 5 min, sub-samples were taken and left to quantify fertilization and the assay was diluted to 50 mL with FSW. After a further 5 min, the eggs were transferred into plastic buckets containing 15 L of FSW and 1 mg/L ethylenediaminetetraacetic acid (EDTA). The buckets were gently aerated and the temperature maintained at 21 ± 2 ^{o}C. After ~48 h, the buckets were drained through a 40-µm mesh and the number of normal D-stage larvae were counted in replicate sub-samples from a homogeneous suspension (Adams et al. 2008a).

Green-lipped Mussels

For mussels, 1 million eggs from each pool were fertilized at a ratio of 5000 cryopreserved sperm/egg and an egg density of 3,000 – 10,000 eggs/mL. After a 20 min contact time, the eggs were transferred to buckets containing 15 L of FSW and 1 mg/L EDTA at ~18 ^{o}C (20 eggs/mL during incubation).

Paua Abalone

Abalone development was assessed using a bulk fertilization method in which a sperm/egg slurry was used to increase fertilization. Eggs were fertilized at a ratio of 10,000 sperm/egg and a density of 25,000 eggs/mL. After a contact time of 10 min, the eggs were diluted in 1 L of seawater and transferred to buckets each containing 15 L of FSW (5-15 eggs/mL during incubation).

Sperm Cryopreservation

A range of factors such as cryoprotective agent (CPA) type, CPA level, combinations of CPAs, diluent composition, sperm:diluent ratios, freezing method and rate, and packaging vessels (straws vs. cryovials) were tested using the fertilization assays. These trials involved a minimum of three different batches of sperm and eggs for each treatment.

Pacific Oyster

The technique developed for cryopreservation of Pacific oyster sperm has been documented (Smith et al. 2001, Adams et al. 2000b, 2004, 2008a) and has also proved successful for sperm of Sydney rock oyster *Saccostrea glomerata* (Adams 2005, 2007; O'Connor 2007). The current Cawthron oyster sperm method is summarized as follows:

1) A stock trehalose solution is prepared by adding 15.2 g of trehalose to 40 mL of Milli-Q water. Trehalose stock solution (30 mL) is combined with 7.8 mL of Milli-Q water and 2.2 mL of dimethyl sulfoxide (DMSO) to prepare the CPA solution which is cooled on ice.
2) A methanol/dry ice bath is prepared by placing several L of methanol and dry ice into a polystyrene box. The bath should be prepared in advance to ensure it has cooled to below -70 °C before use. As a guide, the bath is sufficiently cold if there is dry ice left, but bubbling and gas release has reduced to a minimum.
3) Sperm is stripped from mature males and held "dry" on ice until ready for use. The concentration of the collected sperm is determined.
4) The sperm is diluted 1:10 with CPA solution. The solution should be added to the sperm in 10 steps, 10 to 20 sec apart with mixing between each step, to reduce osmotic shock. The sperm mixture is loaded into chilled 4.5-mL cryotubes with internal threads (Nalgene, Nunc International, Denmark) and attached to aluminium canes (for ease of handling and storage). The canes are placed with the cryotubes directly into the methanol/dry ice bath. After 10 min, the canes are transferred quickly from the bath into liquid nitrogen.
5) The samples are thawed in a lidded container of seawater at ~20 ^{o}C until the contents become liquid (5 to 8 min), and used for fertilization.

The motility and viability of cryopreserved sperm was reduced compared to fresh sperm, but fertility remained adequate. Typically a 10-to-100 fold higher sperm-to-egg ratio was required with cryopreserved sperm for fertilization equivalent to that of fresh sperm (Figure 3).

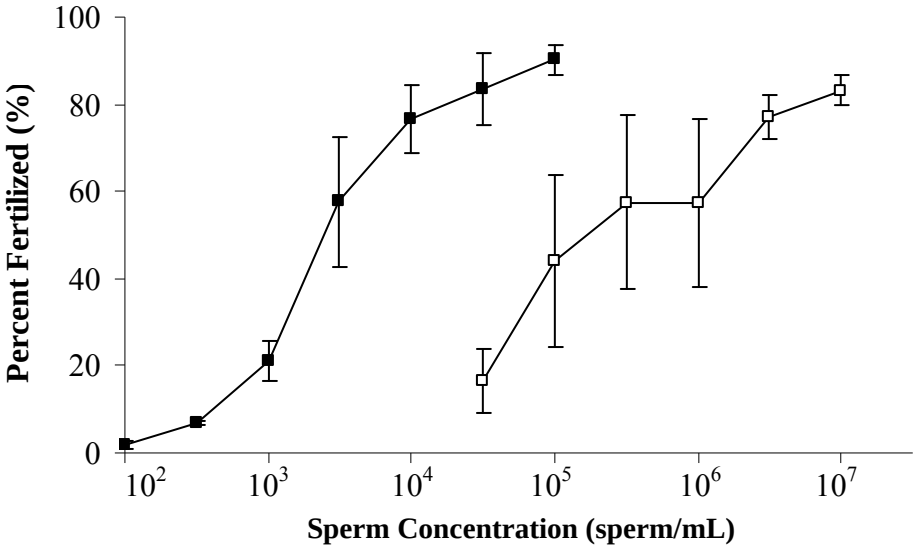

Figure 3. Fertilization (mean ± SEM) of eggs using fresh (closed squares) or cryopreserved (open squares) Pacific oyster sperm. Three pools of sperm were collected, cryopreserved and assayed independently. The same batches of eggs were used with the fresh and thawed sperm. (Modified from Adams et al. 2004).

The method was repeatable in terms of fertilization (Figure 4) and D-stage larval yields (Pool A: 85 ± 3% (mean ± SD); Pool B: 82 ± 2%).

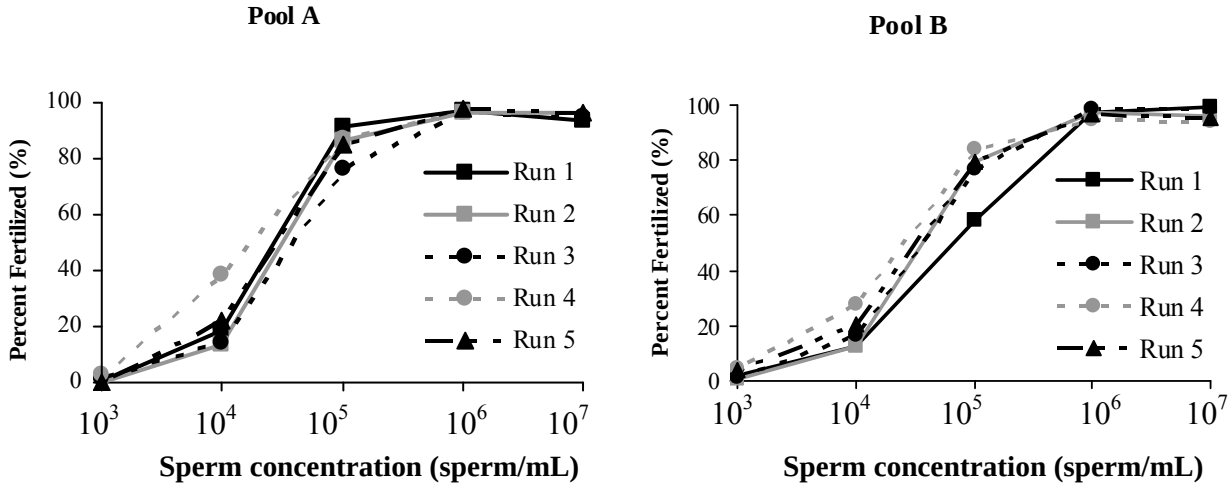

Figure 4. Fertilization rates obtained from five independent experimental trials for each of two pools (A and B) of cryopreserved Pacific oyster sperm (modified from Adams et al. 2008a).

Green-lipped Mussel

Experimentation similar to that described for oysters was undertaken with mussel sperm. DMSO was the most effective cryoprotectant at a final concentration of 12%. Further experiments indicated that the addition of trehalose at a low concentration (0.25 M) was beneficial (Adams et al. 2008c) (Figure 5).

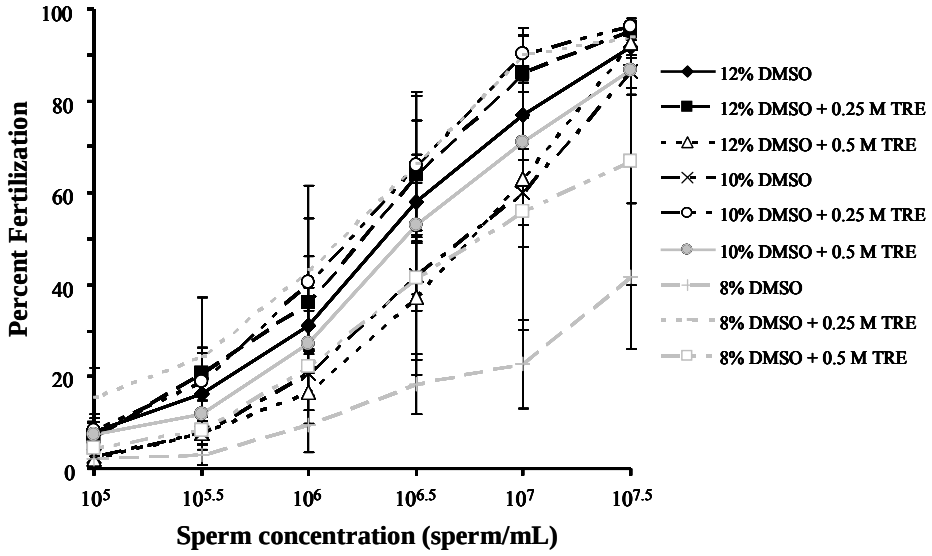

Figure 5. Fertilization curves showing the effect of DMSO and trehalose (TRE) combination on post-thaw fertility of green-lipped mussel sperm (mean ± SEM) (n = 3 pools of sperm).

The lower concentration of mussel sperm resulted in a dilution rate of sperm to CPA solutions of 1:1 being the most appropriate. This lower dilution rate and the smaller volumes of mussel sperm collected means that 0.5-mL plastic straws sealed with colored PVC powder (Instruments de Médicine Vétérinaire, l'Aigle, France) rather than cryotubes were the preferred packing option. The most practical freezing method was the use of liquid nitrogen vapor in the "rack system" where a stainless steel rack was attached to a polystyrene rectangular frame that floated on liquid nitrogen and held the straws 3 cm above the surface of several L of liquid nitrogen in a polystyrene box. After the straws were placed on the rack, a lid was placed on the box and the samples remained on the rack for 10 min before being plunged into liquid nitrogen. This method gave fertility equivalent or slightly better than that obtained with a programmable freezer cooling at a rate of 4 ^{o}C/min.

However, post-thaw fertility remained extremely variable among mussel sperm from individual males and pools of different males (Smith et al. 2006) (Figure 6). Research is on-going towards improving post-thaw fertility and larval yield so that cryopreservation can be routinely applied in mussel selective breeding. One approach is to use motility stimulants.

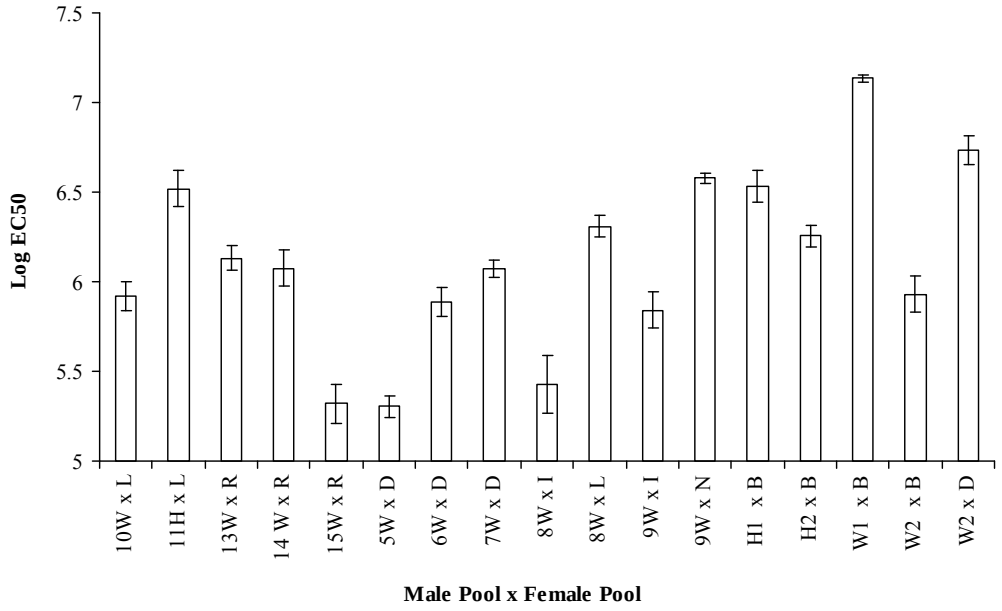

Figure 6. Variation in post-thaw fertility among pools of green-lipped mussel sperm presented as the mean log concentration of sperm required to achieve a 50% fertilization (EC50) ± SEM.

Pentoxyfylline and caffeine were evaluated at a range of concentrations (1-20 mM). The stimulants had little effect on post-thaw motility scores; however fertilization markedly increased at the lowest concentration (1 mM). This improvement resulted in a 10-fold reduction in the concentration of thawed sperm required to achieve 50% fertilization (Figure 7) (Adams 2008c).

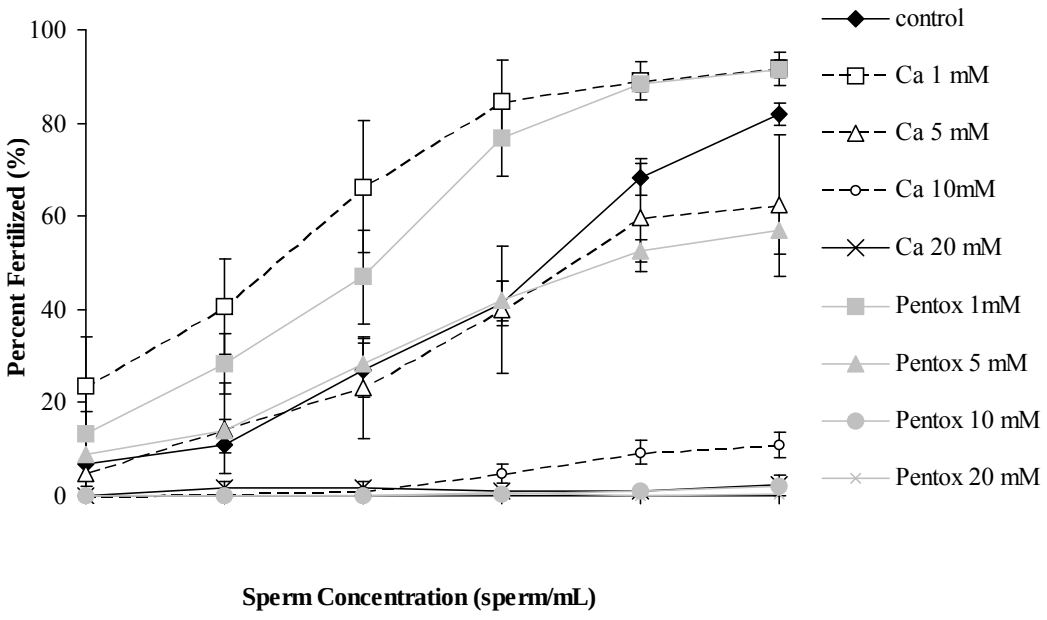

Figure 7. Fertilization curves showing the effect of the motility stimulants, caffeine (Ca) and pentoxyphylline (Pentox) at various concentrations on post-thaw fertility of green-lipped mussel sperm (mean ± SEM) (n = 3 pools of sperm).

Research is being conducted to determine if these stimulants affect embryo development. The current Cawthron method for cryopreserving mussel sperm is summarized as follows:

1) A stock trehalose solution is prepared by adding 15.2 g of trehalose to 30 mL of Milli-Q water. Trehalose stock solution (15 mL) is combined with 7.8 mL of Milli-Q water and 7.2 mL of DMSO to make the CPA solution which is cooled on ice.
2) Mussels are induced to spawn using thermal cycling. Sperm is collected "dry" every 30 min and stored at 5 ^{o}C before pooling.
3) A liquid nitrogen bath is prepared for use in freezing sperm by placing several L of liquid nitrogen into a polystyrene box.
4) Sperm are diluted 1:1 with CPA solution. The solution is added to the sperm in 4 steps, with each step 30 sec apart and mixing occurring between each step to reduce osmotic injury. The sperm mixture is loaded into pre-labelled 0.5-mL plastic straws. Straws are sealed with colored PVC powder and placed on a stainless steel rack attached to a polystyrene rectangular frame.
5) The frame with rack is floated on the surface of the liquid nitrogen so that the straws are 3 cm above the surface of the liquid nitrogen. The lid is placed on the polystyrene box. After 10 min the straws are plunged into liquid nitrogen.
6) The straws are thawed in a container of seawater at room temp (~18 ^{o}C) until contents become liquid (10 - 20 sec) and are used for fertilization.

The motility and viability of cryopreserved sperm was reduced compared to fresh samples (Smith et al. 2000), but fertility remained adequate. Typically the sperm-to-egg ratio required to achieve a given percent fertilization was 10- to 100-fold higher with cryopreserved sperm than with fresh sperm (Smith et al. 2006).

Paua Abalone

Again, DMSO appeared to be the most suitable of the membrane permeable CPAs used (Adams et al. 1999, Roberts et al. 2000b) but the use of non-permeable sugars alone was also successful (Figure 8) (Smith et al. 2006, Adams et al. 2007).

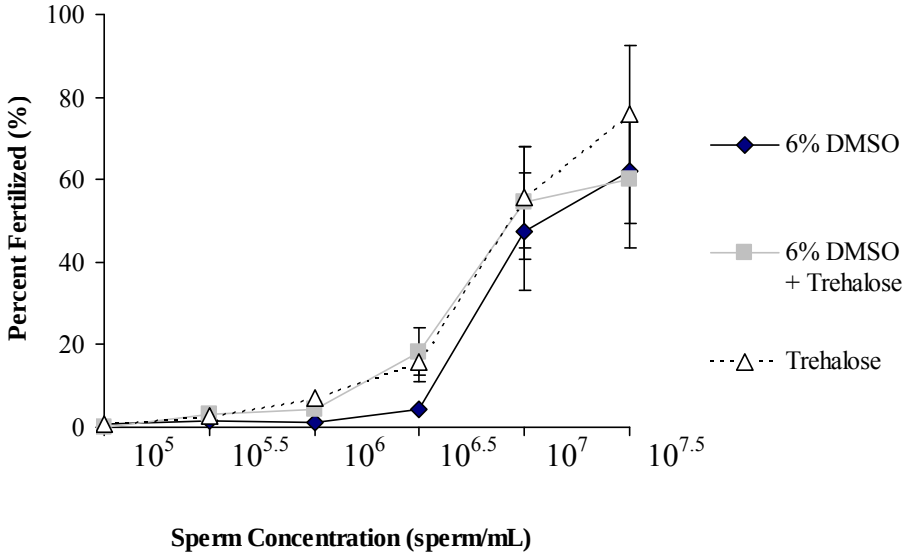

Figure 8. Comparison of DMSO and trehalose, alone and in combination on post-thaw fertility of Paua abalone sperm (mean ± SEM) (n = 3).

A wide range of sugars (e.g., sucrose, glucose, fructose, maltose, and trehalose) have been used at a range of concentrations but best results have been obtained with sucrose at 0.8 M (Figure 9).

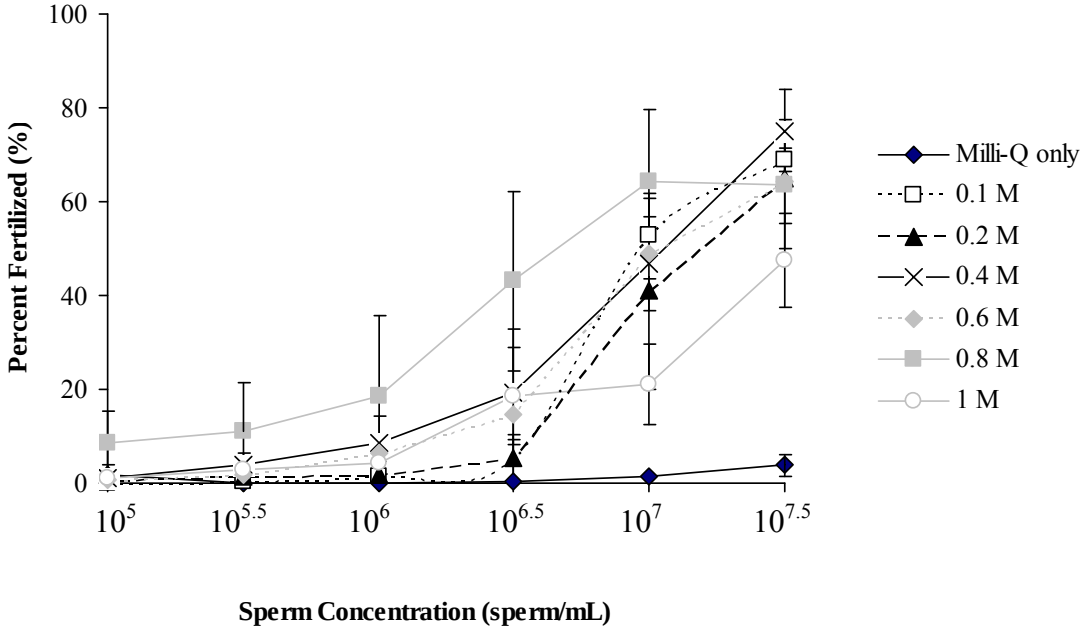

Figure 9. Effect of different concentrations of sucrose as the sole CPA on post-thaw fertility of Paua abalone sperm (mean ± SEM) (n = 3).

Currently the most successful methods for freezing abalone sperm are the methanol/dry ice slurry or programmable freezer systems. The current Cawthron abalone sperm cryopreservation method is summarized as follows:

1) A 1.6 M solution of sucrose is prepared in Milli-Q water and cooled on ice.
2) A methanol/dry ice bath is prepared by placing several L of methanol and dry ice into a polystyrene box.
3) Spawning is induced in dark conditions using chemical stimulation by immersion in seawater containing 250 ppm hydrogen peroxide and 1.3 mM NaOH.
4) Sperm is collected "dry" and held on ice until needed. Concentration is determined for collected sperm.
5) Sperm is diluted 1:1 with sucrose solution in 4 steps, with each step 30 sec apart. Sperm mixtures are loaded into 0.5 mL straws and sealed with colored PVC powder.
6) Straws are loaded into programmable freezers (Cryologic Pty Ltd., Mt. Waverley, Australia) which are programmed to hold at 0 °C for 5 min, cool at 5 °C/min to -75 °C and hold for 10 min. The straws are plunged into liquid nitrogen and stored.
7) Straws are thawed in a container of seawater at ~14 -18 ^{o}C until the contents become liquid (20- 40 sec) and samples are used for fertilization.

Summary and Conclusions

Cryopreservation methods for the sperm of three species of shellfish have been described that are simple, practical, and effective. The methods have been shown to be repeatable and have been applied to commercial selective breeding programs for two of these species (oyster and mussel).While the methods are basically similar, for optimum results, there are differences in

detail between the three species. In addition, there remains a large unexplained variability in the post-thaw fertility of sperm between different males of all three species. These between and within species differences have also been reported for mammalian species (Holt 2000). Further research is required to devise treatments that can optimize the fertility levels and larval yields of those males that yield sub-optimal post-thaw fertility.

Acknowledgments

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New Chapter

Cryopreservation of Macha Surf Clam Spermatozoa

Enrique Dupré and Alicia Guerrero

Introduction

The macha surf clam *Mesodesma donacium* is one of the most valuable bivalve mollusks for fishing communities of the Pacific coasts of Chile and Perú. It is a diecious species without sexual dimorphism. It lives buried in sub-tidal sand beaches and spawns mainly during the spring and summer (October to March in Chile) (Osorio 2002). This clam is imperiled due to severe exploitation during the last 10 yr and climatic changes. Complete disappearance of populations occurred from the shores of Coquimbo in Chile to the southern shores of Perú (Miranda 2001) which was attributed to high mortality due to changes in the environment (i.e., low salinity conditions and high frequency of storms with swells) generated by the "El Niño" event of 1997-1998 (Arntz et al. 1987). This disappearance of the "macha" bank was not only an ecological disaster, but an economic and social problem for the local fishermen (Ortiz and Stotz 2003, Aburto and Stotz 2003). One approach to address the instability of this species is gamete cryopreservation and long-term storage to preserve stocks for future use. That is why our laboratory initiated studies to cryopreserve gametes of macha. The overall goal of this work was intensive production of larvae by use of cryopreserved sperm. To achieve this goal, we focused on improving the survival of sperm after cryopreservation and on improving fertilization by using high concentrations of thawed sperm.

Initial Cryopreservation Studies

We evaluated the pre-freeze toxicity of three cryoprotectants: dimethyl sulfoxide (DMSO), methanol (MeOH) and propylene glycol (PG) as a preliminary step for cryopreservation (Joo and Dupré 2002). With an equilibrium time of 5 min, 1 M DMSO yielded the highest percent motility. To determine the best cryopreservation protocol, a combination of the three cryoprotectants, with three molarities (0.5 M, 1.0 M, and 1.5 M), four cooling rates (5, 10, 15, and 20 °C/min), and two thawing rates (72 and 312 °C/min) were evaluated (Dupré and Joo 2006). Sperm motility and fertilization were used to estimate survival of sperm after thawing. The best fertilization percentage (84%), with a motility of 12%, was obtained with 1.5 M DMSO, a cooling rate of 15 °C/min and a thawing rate of 312 °C. A higher motility (17%) was observed following the same protocol with a thawing rate of 72 °C, however, this did not yield the best fertilization (62%). To optimize cryopreservation we evaluated two different techniques in subsequent studies: cryopreservation of stripped sperm and of pieces of testes.

Cryopreservation Optimization Studies

Animal Collection

Macha surf clams were collected from the beach at Coquimbo, Chile, by local fishermen and transported to the Marine Science Faculty hatchery in the Universidad Católica del Norte, Coquimbo, Chile. After 2 d of acclimatization in an open laboratory system, the clams were

Dupre, E. and A. Guerrero. 2011. Cryopreservation of Macha Surf Clam Spermatozoa. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 574-580.

opened and visually inspected to detect maturity as a prominent gonadal area, and the presence of eggs or sperm. The surface of the gonad was sliced (Figure 1) such that gametes could be collected with a Pasteur pipette for examination at 100-x magnification.

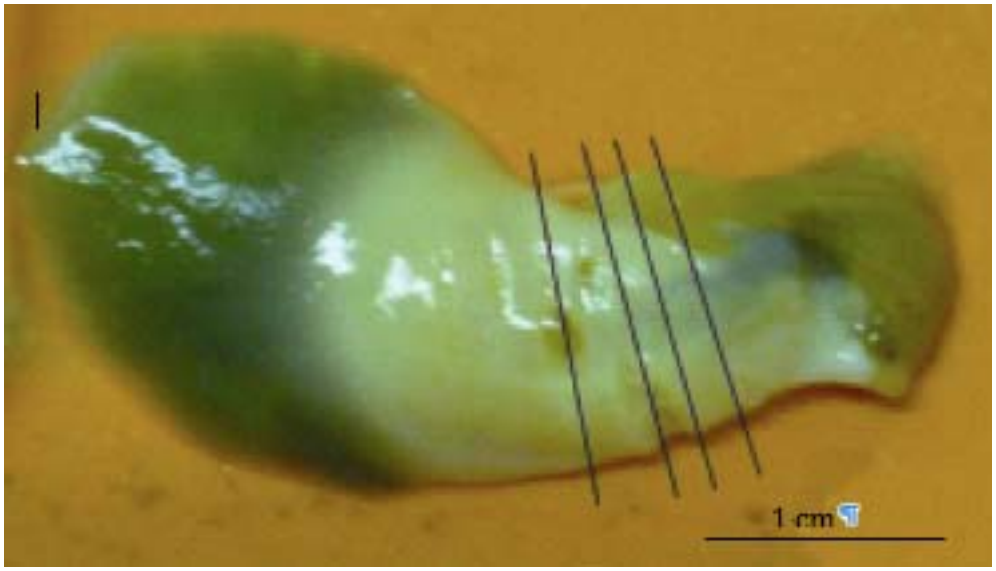

Figure 1. Macha surf clam body showing position of the slices made on the gonad to obtain gametes by stripping.

Gamete and Gonad Preparation

Spermatozoa were stripped (SS) from the testes after superficial cutting with a scalpel, and collected by a Pasteur pipette and suspended in filtered sea water or cryoprotectant. For the collection of gonads, pieces of testes were gently disrupted into two size ranges: medium-sized pieces (MP) 0.25 – 0.28 g, and small pieces (SP) 0.18 – 0.20 g.

For females, the oocytes were collected by stripping and placed in separate 50-mL beakers, suspended in filtered seawater, and washed twice with filtered seawater to be fertilized with stripped sperm or sperm from the pieces of testes.

Freezing and Thawing Procedures

As mentioned above, two different techniques were evaluated for cryopreservation of gametes (stripped sperm) and cryopreservation of gonads (pieces of testes). All samples were pooled from 3 individuals and placed into 1.5-mL cryovials containing different cryoprotectants with different concentrations.

The cryoprotectants DMSO, ethylene glycol (EG) and PG were used at concentrations of 0.5 M, 1 M, 2 M, or 3 M, prepared with fresh filtered seawater, with addition of 5% sucrose and 10% egg yolk. After mixing, the cryoprotectant solution with the sperm samples or pieces of testes were equilibrated for 10 min at 5 °C. After equilibration, the samples were cooled in a controlled-rate freezer (Planer Kryo 560-16) in a single step, from 5 °C to -80 °C at cooling rates of 5, 8, 12, 15, 18, and 20 °C/min. After reaching a final temperature of -80 °C, the cryovials were held for 5 min, plunged into liquid nitrogen and stored in a Dewar for 5-14 d. The thawing rates were 72 °C/min (slow) and 312 °C/min (fast) obtained by immersing the cryovials in a water bath at 50 °C for 20 sec or 30 °C for 40 sec, and by later allowing the cryovials to warm at

room temperature (20 °C) for 4 min. The post-thaw sperm motility and the percentage of fertilization were evaluated.

Fertilization

The thawed pieces of testes were washed in filtered sea water and agitated to obtain a highly concentrated sperm solution to fertilize fresh oocytes. Sperm concentration was estimated by image analysis. One drop (60 μL) of thawed sperm was diluted in 3 mL of seawater, and placed on a Neubauer chamber to determine the sperm motility, which was recorded by 4 videos of 5 sec with a digital camera (Canon PowerShot A620) and stored digitally for later analysis (speed and moving distance) using the software Image ProPlus 5.1.

A sample of 50 mL of fresh oocytes (3,000 – 5,000 oocytes/mL) was placed in a 100-mL capsule and fertilized with different sperm concentrations (15 – 40 $\times 10^6$ sperm/mL) to yield a final ratio of 1,000 sperm/oocyte. Assessment of fertilization was done after 10 min by recording the percentage of morulae obtained. As a control, pooled fresh oocytes were fertilized with pooled fresh sperm from new sets of three males and females.

The values for average speed and fertilization obtained with thawed sperm were arc-sin square root transformed and analyzed by a two-way ANOVA and Tukey's test. The fertilization percentages of the control varied between 62 ± 5% and 100%. However, for statistic analysis, the percent fertilization of the controls for each protocol were considered to be 100%, and the experimental percentages were calculated in relation to the control values for each protocol.

Results

Fertilization Rates

Large differences were observed in the fertilization obtained with thawed spermatozoa from different individuals. Overall, cryopreserved spermatozoa collected by stripping yielded lower fertilization rates (40%) compared with sperm collected from the small pieces of cryopreserved testes (fertilization between 97% and 88%) (Figure 2). No significant differences were observed between small-size pieces and medium-size pieces of testes (Figure 3, next page).

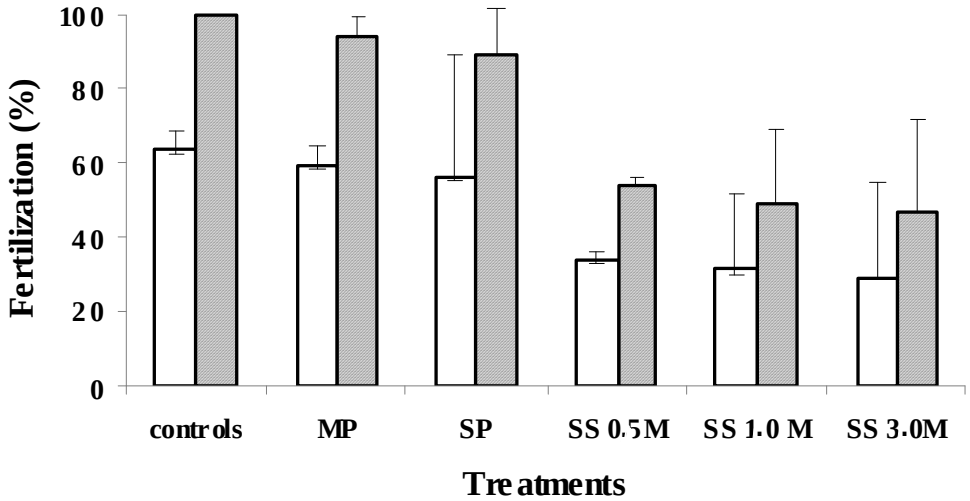

Figure 2. Absolute fertilization (white bars) and values relative to control (filled bars) obtained with thawed spermatozoa from mid-size pieces (MP) and small pieces (SP) of testis, and sperm suspended (SS) in cryoprotectant solution (DMSO with sucrose and egg yolk).

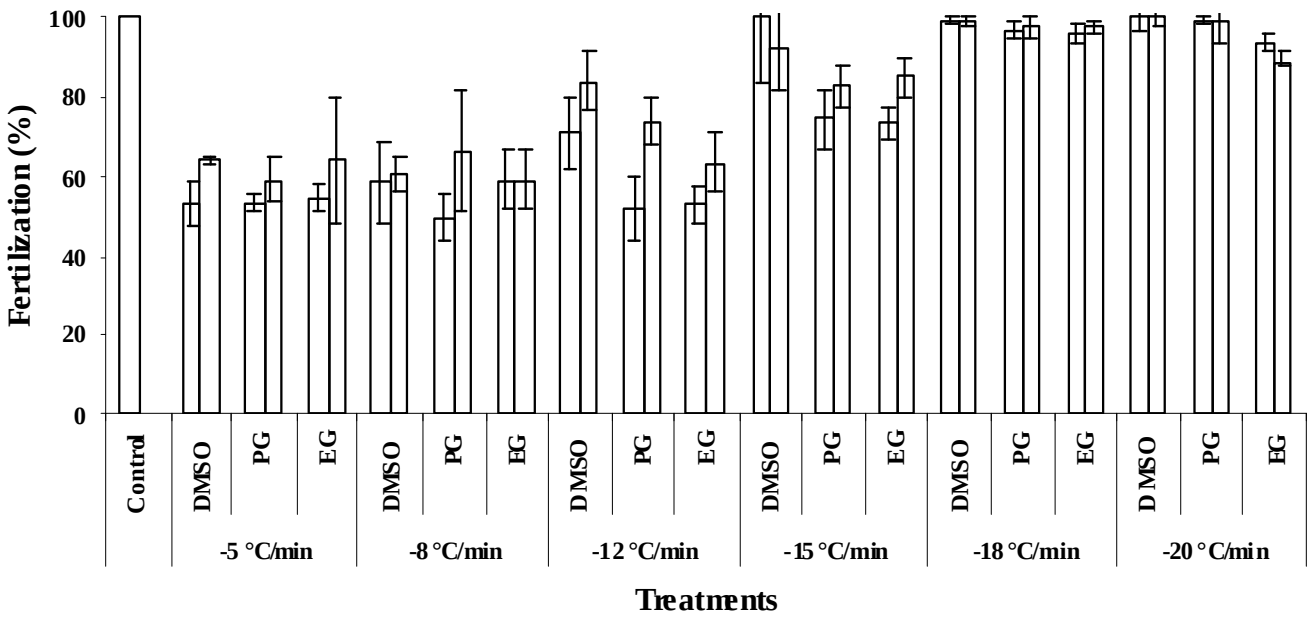

Figure 3. Fertilization of fresh oocytes inseminated with thawed spermatozoa which were frozen as small pieces (filled bars) or medium pieces (white bars) of testis at different cooling rates, at 3 M concentration of different cryoprotectants. The controls (ranging from 52% to 91%) were adjusted to 100% for comparison.

Cryoprotectants

Fertilization rates obtained from spermatozoa extracted from thawed pieces of testes with different cryoprotectant were significantly different ($P < 0.05$) (Figure 4).

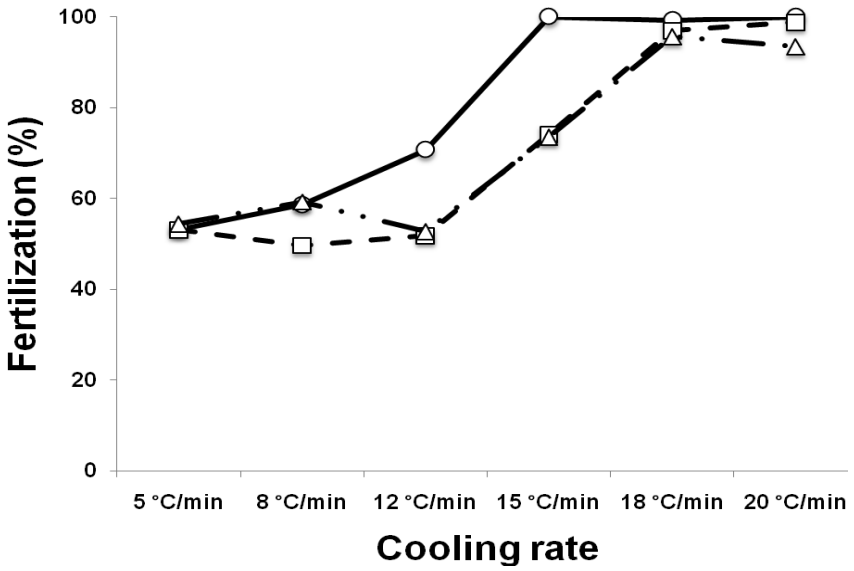

Figure 4. Fertilization obtained with spermatozoa subjected to different freezing rates using different cryoprotectants (DMSO circles; PG squares; EG triangles) at 3 M concentration.

The best fertilization percentages (97% ± 2%) were obtained when DMSO was used, while the lowest fertilization was obtained with EG. When PG or EG were used, no significant differences were observed between fertilization percentages (63 ± 18% and 60 ± 17%) (Figure 4), but the differences between each of these cryoprotectant and DMSO were significant ($P < 0.05$). Although the best fertilization percentages were obtained with 1 M DMSO, there were no significant differences among the three concentrations tested.

Cooling and Thawing Rates

The best fertilizations were obtained with cooling rates of 18 ºC/min and 20 ºC/min (Figures 4 and 5). The highest fertilization percentage (97 ± 2%) was obtained with a freezing rate of 18 ºC/min using 2 M DMSO as cryoprotectant, while the lowest (48 ± 4%) was with 8 ºC/min using PG. When increasing the cooling rate, a tendency to increase the fertilization percentages was observed, mainly when DMSO was used (Figure 5). When using EG or PG, the best cooling rate was 18 ºC/min. The thawing rates were not significantly different, although the best fertilization results were obtained with a fast rate.

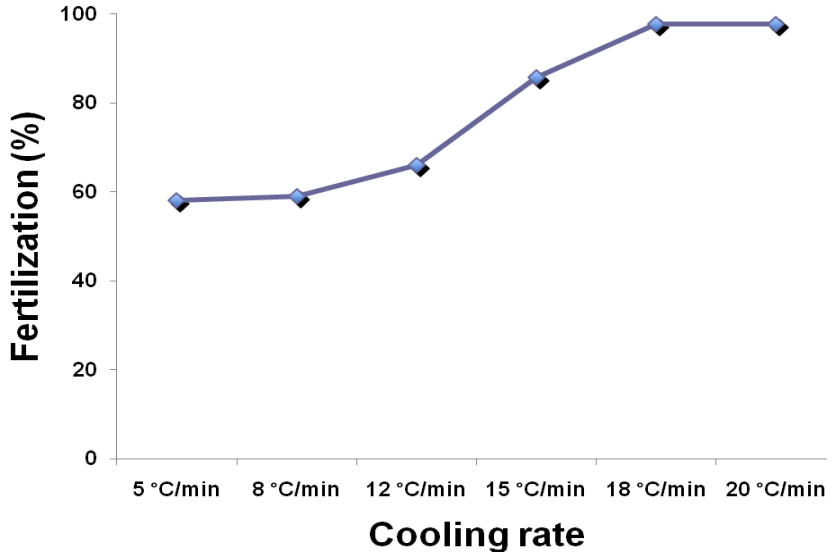

Figure 5. Fertilization (relative to control) of fresh oocytes with thawed spermatozoa frozen with 2 M DMSO at different cooling rates.

Discussion

Sperm obtained from cryopreserved pieces of testes yielded fertilization percentages about 2 times higher than those obtained with cryopreserved stripped sperm. When different cooling rates were tested, the highest fertilization was obtained with the protocols that included DMSO. A similar fertilization percentage (84%) were obtained for this species (Dupré and Joo 2006) at a cooling rate of 15 ºC/min, using DMSO as cryoprotectant, which coincided with the fertilization percentage obtained at 15 ºC/min in the present study (80%). However, the present study obtained better fertilization percentages (97 ± 2%) at 18 ºC/min and 20 ºC/min.

One potential benefit of the results obtained in this study, given that sperm can be cryopreserved within pieces of gonad, is that these results can be applied to hatchery production of larvae, because the numbers of sperm retrieved from these pieces of testicular tissue were

greater than those obtained by stripping of the gonad. These results open the possibility of mass production of larvae, and the possibility to initiate culture of this species, which until now has not been attempted.

The high fertility rates obtained by insemination with cryopreserved sperm from thawed testicular pieces can be explained in two ways: 1) the number of sperm obtained from testicular pieces was larger than the number obtained from stripping, or 2) a molecule or substance present in the testis protects the sperm during freezing. We obtained a significant increase in the percentage of motility and movement speeds when homogenized material from testis was added to cryoprotectant solutions (unpublished data).

Sperm concentration affected fertilization rates in cryopreserved sperm of small abalone (*Haliotis diversicolor supertexa*) which varied between 7 and 48%, and increased as concentration increased (Gwo et al. 2002). This agreed with our results that sperm from pieces of testes generated higher percent fertilization compared with sperm solutions. The sperm numbers obtained by this method were greater than these obtained by spawning or stripping of the gonad. These results show that sperm can be cryopreserved in large quantities within small pieces of gonad, which allows mass spawning in hatcheries to obtain large numbers of larvae for culture.

Acknowledgments

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The Need for Standardization in Cryopreservation: a Case Study with Oysters

Qiaoxiang Dong, Changjiang Huang and Terrence R. Tiersch

Introduction

Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to very low temperatures, typically, -196 °C (the boiling point of liquid nitrogen), yet remain viable after subsequent warming to temperatures above 0 °C. For sperm cryopreservation, this process typically includes gamete collection, suspension of sperm in an extender, quality assessment, addition of cryoprotectants, equilibration, freezing, thawing and fertilization, and subsequent development of early life stages for assessment of cryopreservation success (Tiersch 2000). The term "extender" refers to a solution of salts, sometimes including organic compounds such as sugars that helps maintain sperm viability prior to and during the freezing process. This term has also been used to include cryoprotectant molecules (e.g., dimethyl sulfoxide or methanol) in some literature; however, its use in this chapter refers to salt solutions only (e.g., calcium-free Hanks' balanced salt solution).

Numerous studies in sperm cryopreservation have been devoted to optimizing specific components of cryopreservation procedures. However, aside from those factors mentioned above, other factors such as sample density, freezing container, starting temperatures, final temperatures (before plunging into liquid nitrogen), and dilution and cryoprotectant removal after thawing could also affect results (Leibo 2000). This review calls attention to the importance of the cumulative and interacting effects arising from all activities in the cryopreservation process. The remainder of this chapter is intended to provide an overview of sperm cryopreservation in oysters with an emphasis on identifying problems, variation, and lack of standardization among previous studies. This review also points out where and how this research differs from previous studies, and the importance of standardization for the future potential commercialization of cryopreserved sperm in aquatic species.

A Case Study: Sperm Cryopreservation in Oysters

The beginning of the science of cryobiology can be traced back to the 1950s after the discovery of the cryoprotective qualities of glycerol for fowl sperm (Polge et al. 1949). The first studies of fish sperm cryopreservation were published 4 yr later (Blaxter 1953), and since then more than 200 fish species have been studied (Rana 1995, Tiersch 2000) although this estimate requires updating. In contrast to the extensive studies in cryopreservation of fish semen, similar work for invertebrates has been limited to echinoderms (sea urchins, sand dollars, and starfishes),

Dong, Q., C. Huang and T. R. Tiersch. 2011. The Need for Standardization in Cryopreservation: a Case Study with Oysters. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 581-593.

mollusks (oysters and abalone), polychaetes, and crustaceans (shrimps and crabs) (e.g., reviewed by Gwo 2000).

Despite the limited work in aquatic invertebrates overall, oysters are well studied reflecting their economic importance around the world. Indeed, the oyster cryopreservation literature provides a strong database to illustrate the types and breadth of problems inherent in the lack of consistency in methods and reporting for cryopreservation in all aquatic species. For the 35-yr period between 1971 and 2006, there were 26 reports directly related to oyster sperm cryopreservation since the first study some 40 yr ago (Lannan 1971). These reports comprised 16 peer-reviewed journal articles, 1 abstract, 2 book chapters, 2 conference proceedings, 1 thesis, 1 dissertation, 1 technical report, and 2 review articles (Table 1, next page). Except for the review articles, 19 of the 24 research reports (~80%) were produced for sperm from the Pacific oyster, *Crassostrea gigas*. These research efforts have yielded techniques with varying levels of success. However, similar to the situation observed with other aquatic species, sperm cryopreservation in oysters has not yet found application in aquaculture on a commercial scale.

As in most other aquatic species, one of the major obstacles to widespread application is the inconsistency of various components of cryopreservation technology among and within studies, such as initial sperm quality, gamete collection methods, extender formulation, cryoprotectant choice, cooling rate and method, thawing rate and method, insemination protocols, and evaluation of post-thaw sperm quality (Rana 1995, Gwo 2000, Tiersch 2000). Lack of procedural standardization in the cryopreservation of oyster sperm is identified in detail in this chapter (see Appendix), but the same problems identified here would be routinely observable in other aquatic species.

Gamete Collection

Cryopreservation of oyster sperm involves many variables from broodstock condition to larval development, and for each step, various procedures have been used among different studies (these are summarized in the Appendix at the end of the chapter). For gamete collection, the two most commonly used methods were dry stripping and aspiration using pipette or syringe. Non-destructive methods (without killing of the oyster), which would be especially useful for the purpose of self-fertilization (Lannan 1971), include withdrawal of gonad material by use of a syringe through holes drilled in the shell, or induced spawning. Few studies have indicated what part of the gonad was sampled, and a recommendation was provided that no more than 50% of the gonad volume should be extracted to avoid including immature or nutritive cells (McFadzen 1995). Samples from individual males or pooled samples from several males were used for various studies. Fewer than half of the reports (excluding the two review articles) indicated a sperm quality assessment prior to freezing, and when assessed, motility was the sole criterion used.

Table 1. Literature published (between 1971 and 2006) on sperm cryopreservation in oysters.

Reference number	Species	Summary of findings	Reference
1	*Crassostrea gigas*	0-10% fertility[a]; 0-3% larvae[a]	Lannan, 1971[1]
2	*C. virginica*	1-5% motility, 11% fertility[a] (2% normal fertility)	Hughes, 1973[1]
3	*C. gigas*	79% fertility[a]	Hwang and Chen, 1973[6]
4	*C. gigas*	Highest mean value: 36% fertility[a], 28% larvae[a]	Staeger, 1974[5]
5	*C. virginica*	7-91% fertility[a]	Zell et al., 1979[1]
6	*C. gigas*	0-26% fertility[a]	Van der Horst et al., 1985[2]
7	*C. gigas*	1-3 (0-5 scale) motility, 13-75% fertility[a], 47-92% fertility[b]	Bougrier and Rabenomanana, 1986[1]
8	*C. gigas*	0-106% fertility[b]	Iwata et al., 1989[1]
9	*C. gigas*	20-30% motility, 23-40% survival (19-57% normal shape)	Kurokura et al., 1990[1]
10	*C. tulipa*	0-71% fertility[a], 0-55% larvae[a], 87-93% survival at day 16.	Yankson and Moyse, 1991[1]
	C. iredalei	11-35% or 1-3 (0-5 scale) motility	Yankson and Moyse, 1991[1]
	C. gigas	48-93% fertility[a], 0-18% larvae[a]	Yankson and Moyse, 1991[1]
	Saccostrea cucullata	0-78% fertility[a], 0-51% larvae[a]	Yankson and Moyse, 1991[1]
11	*C. gigas*	(Protocols only)	McFadzen, 1995[3]
12	*C. gigas*	2-3 (1-4 scale) motility, 18-77% viability, 0-70% fertility[a], 0-70% larvae[b]	Usuki et al., 1997[1]
13	*C. virginica*	0-22% motility, 0-78% larvae[a]	Paniagua-Chavez, 1999[5]
14	*C. gigas*	Same as Number 12.	Usuki et al., 1999[4]
15	Invertebrates	(Review paper)	Gwo, 2000[7]
16	*C. virginica*	8-1316% survival[b] beyond settlement (juvenile)	Paniagua-Chavez et al., 2000[3]
17	Finfish and shellfish	(Review paper)	Chao and Liao, 2001[7]
18	*C. virginica*	Same as Number 13	Paniagua-Chavez and Tiersch, 2001[1]
19	*C. gigas*	0-100% fertility[a]	Smith et al., 2001[4]

Summary of findings: [a]absolute percentage; [b]relative percentage to controls

Report format: [1]journal article, [2]published abstract, [3]book chapter, [4]conference proceedings, [5]thesis or dissertation, [6]technical report, [7]review article.

Table 1 Continued.

Reference number	Species	Summary of findings	Reference
20	*C. gigas*	0-70% motility, 68% fertility[a], 64% hatch[a]	Li et al., 2002a[1]
21	*C. gigas*	(Morphological examination only)	Li et al., 2002b[1]
22	*C. gigas*	2 (0-4 scale) motility, 0-40% fertility[a]	Gwo et al., 2003[1]
23	*C. gigas*	0-90% fertility[a], 12% survival at settlement	Adams et al., 2004[1]
24	*C. gigas* (diploid)	(Theoretical prediction for cooling rate)	He et al., 2004[1]
	C. gigas (tetraploid)	(Theoretical prediction for cooling rate)	He et al., 2004[1]
25	*C. gigas*	0-59% regular D-stage larvae	Ieropoli et al., 2004[1]
26	*C. gigas* (diploid)	0-30% motility, 0-96% fertility[a]	Dong et al., 2005[1]
	C. gigas (tetraploid)	0-15% motility, 0-28% fertility[a]	Dong et al., 2005[1]

Summary of findings: [a]absolute percentage; [b]relative percentage to controls..

Report format: [1]journal article, [2]published abstract, [3]book chapter, [4]conference proceedings, [5]thesis or dissertation, [6]technical report, [7]review article.

Extender Choice

The most commonly used extender (when specified) was sterilized or filtered seawater, followed by artificial seawater. Other extenders included Hanks' balanced salt solution (HBSS), calcium-free HBSS (C-F HBSS), DCSB4, Hanks' phosphate buffer, glucose, polysaccharide, and sodium citrate. The ion concentrations in various extenders (when reported) were expressed as salinity (parts per thousand), strengths (portion), or osmolalities (mOsmol/Kg). The pH values (when reported) ranged from 7.0 to 8.5. Only two or three studies specified the method of extender preparation, storage temperature, and the grade of chemicals used. Refrigerated storage of fresh sperm was evaluated in only two studies with sperm samples either in undiluted or diluted form and stored at 4 °C for 0 to 7 d.

Sperm Concentration

Only four studies explicitly identified the final sperm concentration in each freezing trial (Staeger 1974, Usuki et al. 1997, Paniagua-Chavez et al. 2000, Dong et al. 2005). Most reports indicated the dilution ratio of sperm volume to cryoprotectant solution, of which three reports identified the original sperm concentrations for sampled milt. Final sperm concentrations ranging from 5×10^8 to 1.4×10^9 cells/mL were considered optimal for freezing.

Cryoprotectant and Equilibration Time

Cryoprotectants included dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, propylene glycol, methanol, trehalose, and glycine. Among these, DMSO was the one most commonly used (in 19 reports); the concentrations that were tested ranged from 2.5 to 20% in various studies. The concentration of DMSO that was considered to be optimum varied among studies, ranging from 5 to 20%, with most reports referring to either 8 or 10%. In addition, ethylene glycol at 10%, propylene glycol at 5, 10, or 15%, and trehalose at 0.45 M were also considered in different studies to be effective in maintaining post-thaw fertility. The addition of a cryoprotectant was usually performed in a single step. However, step-wise additions were suggested by some to avoid osmotic injury (e.g., Adams et al. 2004). Temperatures at which samples were equilibrated with cryoprotectant before freezing varied from 0 to 26 °C with time intervals ranging from 0 to 60 min. In general, shorter equilibration was considered to be more effective in retaining post-thaw sperm quality, but long equilibration (e.g., 60 min) with low cryoprotectant concentrations (< 10%) may not decrease percent fertilization (Dong et al. 2005). The longest time reported between gamete collection and freezing was 4 h.

Freezing Methods

For freezing trials, glass ampules, plastic straws, and cryovials were used as freezing containers with volumes ranging from 0.25 mL to 5 mL, but most studies used plastic straws of 0.25-mL or 0.5-mL volumes. Liquid nitrogen vapor was most commonly used to freeze samples, followed by controlled-rate freezers. Other freezing methods included mixtures of methanol and dry ice (-75 °C), deep freezers (-80 °C), commercial dairy bull sperm freezing methods (Dong et al. 2005), and direct plunging into liquid nitrogen. Various rates of success were reported with

each method (Table 1), but comparisons among them were made difficult or impossible due to inconsistency in methods and reporting of other components (e.g., cryoprotectant and concentrations, equilibration time, and thawing methods) of the cryopreservation procedures. The cooling rates of samples frozen in liquid nitrogen vapor were affected by the distance between samples and the surface of liquid nitrogen, the exposure time, as well as the freezing container itself. For liquid nitrogen vapor, cooling rates reported ranged from 4.7 to 114 °C per min in different studies. Differences in freezing containers played an important role in the rate of cooling regardless of the freezing method. For example, when samples in 4.5-mL cryovials were cooled at a desired rate of 50 °C per min using a controlled-rate freezer, that actual cooling rate was 9.5 °C/min (Adams et al. 2004). A wide range of optimal cooling rates (from 6 to 80 °C/min) was reported in various studies.

Storage and Thawing

For storage, frozen samples were stored at -196 °C or lower in most studies except one, in which samples were stored at -170 °C (Staeger 1974), and storage time varied from 5 min to 4 yr before thawing. Sperm of Pacific oysters cryopreserved for 4 yr yielded 78% normal D-stage larvae and no negative effect was found in mean shell length of the larvae at 6 d after fertilization (Usuki et al. 1997). For thawing, samples were placed in a water bath in most studies, but the temperature of the bath varied from 4 to 75 °C. Thawing at higher temperatures (e.g., 60 °C versus 48 °C) was suggested to more effectively preserve the post-thaw fertility for samples in 0.25-mL straws (Zell et al. 1979) and 5-mL macro-straws (Paniagua-Chavez et al. 2000) of *C. virginica*. Studies with *C. gigas* indicated no difference between thawing at 20 °C for 15 s and at 75 °C for 2 s for samples in 0.25-mL straws (Smith et al. 2001). Incomplete thawing of samples in 1-mL cryovials in 16-17 °C running water followed by a complete thawing at 0 to 4 °C for 10 to 14 min was found to retain higher post-thaw motility (> 40%) in *C. gigas* (Li et al. 2002a). Samples thawed in the air (21 to 22 °C) were considered to be sub-optimal (Staeger 1974). Only one study reported the warming rate, but no thawing method was specified (Ieropoli et al. 2004). Few studies reported dilution or serial dilutions for thawed samples.

Post-thaw Sperm Quality Assessment

Various criteria were used to estimate post-thaw sperm quality, but specific terms had different meanings in different studies. For example, what was defined as percent fertilization in one study (Gwo et al. 2003) was defined as percent hatch in other studies (Staeger 1974, Yankson and Moyse 1991, Dong et al. 2005). Motility was also expressed in several methods such as percentage, or scales of 0–4, 1–4, or 0–5 in increments of 0.5 or 1.0. Similarly, percent survival or viability referred to results derived from different assays. In addition, results for percentage fertilization, larvae produced, and survival were reported as absolute values or as values relative to controls (Table 1). Fertilization methods used in various studies were also different from one another in many aspects, such as sperm-to-egg ratio, the use of eggs from individual females or pooled eggs from several females, scale of the trials (12-well tissue culture plates versus 500-mL plastic beakers), and different types of control treatments. Despite these

differences, for sperm-to-egg ratios, generally a 100-fold increase was suggested for cryopreserved sperm compared to fresh controls (Iwata et al. 1989, Gwo et al. 2003, Adams et al. 2004). Larval development beyond the settlement stages (cessation of the planktonic existence of larvae by attachment to suitable substrates) was also evaluated in six studies, but no adverse effects were reported for larvae produced with cryopreserved sperm.

Summary and Future Outlook

As shown in this review of sperm cryopreservation in oysters, a considerable and potentially troublesome lack of standardization was observed in methods and reporting for each step involved in the cryopreservation process. Comparisons among different studies were difficult to perform and could well be invalid in most cases due to the procedural and reporting variations across studies observed at each step. Studies utilizing the sperm agglutination phenomenon (Dong et al. 2007) clearly demonstrated the requirement for researchers to standardize sperm concentration and methods for oysters (and by extension, all aquatic species) during cryopreservation. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Controversy and inconsistency would be reduced if more congruent approaches were utilized and results among various studies could be directly compared. Suggestions for improvement include the creation and widespread acceptance of standard reference works to assist in harmonizing terminology, and the development and utilization of congruent educational programs. Standardization of research practices and reporting could be facilitated through establishment of guidelines for publication of results. Once in place the guidelines could be made available to journal editors and reviewers to assist in evaluation of research reports.

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Appendix. Variation in methods and reporting for cryopreservation of oyster sperm.

1. Broodstock condition	
Age	11-month-old[1], 1-3 yr-old[12], 2-3 yr-old[26],
Nutrient status	High food rations (mixed algal diet)[11]
Environmental conditions	Loosanoff and Davis method [2, 4], warm water[11], 18-20 °C[16], 25ppt and 20 °C[22],
Gonad maturity	Mature gamete[4, 22, 25], presence of prominent genital canals[13, 16, 26]
Seasonality	Reproduction season[19], January-September[22], November-December[23], August [24], April-August[26]
2. Gamete collection	
Collection methods	Withdrawn by syringe without killing[1, 4,] Spawned[2, 5, 19] Aspiration: pipette[5, 8, 10, 11, 20], syringe[6], Extracted by pressure on the genital gland[7] Chopping of gonad[9, 12, 22] Strip spawning[19, 23] Dry stripping[13, 16, 24, 26]
Part of gonad	Posterior-dorsal region of the right test (flat side)[4] No more than 50% gonad volume[11]
Milt pooled or not	Pooled[4, 5, 7, 8, 23, 25], Not pooled[1, 5, 11, 13, 22, 26]
Quality (threshold)	Motility: (Intensely active)[4, 23], (≥ 4 in 0-5 scale)[7, 10, 25], (> 80%)[9], (> 90%)[13]
3. Shipping	Intact oyster[13, 20, 24, 26]
4. Extender	
Artificial seawater (ASW)	(Not reported)[6], (22, 200, 203, 403, 602, **833** mOsm/kg)[13], (833 mOsm/kg) + 6% glycine[13], (1100 mOsm/kg)[22], (1/2, **2/3**, 5/6, and full strength)[8], (2/3 strength = 670 mOsm)[9, 12], (2/3 strength) + 50 mM sucrose + 6 mM reduced glutathione[12], (2/3 strength) + 36 mM sucrose + 4.3 mM reduced glutathione + 20% FBS[12]
Sterile seawater (SSW)	(25 ppt) [4, 22], (not reported)[1, 7, 20], (34 ppt)[25], (32 ppt) + 0.6% glycine[10]
Seawater (SW)	(not reported)[2, 3, 19]
DCSB4	(not reported)[7], (833 mOsm/kg) [13]
C-F HBSS	(475-679, 830 mOsm/kg)[13, 16], (671, **1000** mOsm/kg)[24, 26]
HBSS	(830, 833 mOsm/kg)[13]
Hanks' phosphate buffer	(13/5 strength)[8], (2.6 strength) + 80 mM glycine + 55 mM NaHCO[5],
Glucose	(0.2, 0.4, 0.6, 0.8, 1M)[8]
Polysaccharide	(not reported)[19]
Sodium citrate	(0.1, 0.15,0.2, 0.25M)[8]
pH	7.0[2], 7.0-8.0[4], 8.0[5], 8.5[7], 7.6[13], 8.2[22]
Preparation	Freshly made[7, 13], 2 h before use[11]
Storage temperature	4 °C[7], 25 °C[11]
Chemical source	Reagent grade[13, 26]

Numbers in superscripts indicate the reference number assigned in Table 1.

Numbers in bold identify optimized protocols of that study.

Appendix. Continued.

5. Refrigerated storage	Undiluted[13, 26], Dilution ratio of sperm to extender (**1:0**, 1:1, 1:3, 1:7, 1:17, 1:31)[13]
Temperature (time)	4 °C (0-4 d)[13], 4 °C (7 d)[26]
6. Sperm concentration	
Initial concentration (cells/mL)	(**2.7 x 10^9**, 3.0 x 10^8, 5.7 x 10^7)[4], (4.78 x 10^{10})[6], (2 x 10^{10})[19], (2 x 10^6, 2 x 10^7, 2 x 10^8, **2 x 10^9**)[26],
Dilution ratio (sperm to cryoprotectant, v/v)	(1:2, 2:1, 1:1)[4], (1:6)[5], (1:25)[6], (1:5, 1:10, **1:12.5**, **1:15**, 1:17.5, 1:20)[7], (1:8)[10], (1:1)[11], (**1:4**)[12], (1:1)[13], (1:1, **1:10**, 1:20)[19], (1:10)[23], (1:10)[25], (1:1)[26]
Freezing concentration (cells/mL)	(5 x 10^9)[8], (**5 x 10^8**, 5 x 10^7, 5 x 10^6)[12], (1 x 10^9)[16], (1 x 10^6, 1 x 10^7, 1 x 10^8, **1 x 10^9**)[26]
7. Cryoprotectant (CPA) and equilibration	
Dimethyl sulfoxide:	(20%)[1], (5, 10%)[2], (3.3, 5, 6.6, 7.5, 15, 20%)[3], (5, **10**, 20%)[4], (**8%**)[5, 12, 26], (6, **9**, 12%)[6], (10%)[7], (4, 6, **8**, 10, 12, 16%)[8], (6, **8,** 10 ,12%)[9], (5, **10, 15, 20%**)[10], (**5**, 10, 15%)[19], (8, **10**, 12, 14, 16, 18, 20%)[20], (**10%**)[21, 22], (2.5, 5, 7.5, 10, 12.5, 15%)[23], (5, 10, 15%)[25]
	(10% + 1 M trehalose)[11], (2.5, **5**, 7.5, 10, 12,5, 15% + 0.45 M trehalose)[23], (5% + 0.55 M trehalose)[23],
Glycerol	(3.3, 5, 6.6, 7.5, 15, 20%)[3], (5, 10, 20%)[4], (5, 10, 15%)[6], (5, 10, 15%)[25], (not reported)[19]
Ethylene glycol	(4, 6, 8, 10, 12, 16%)[8], (5, **10**, 15%)[25], (not reported)[19]
Propylene glycol	(5, **10**, 15, 20, 15%)[13], (5, 10, 15, 20, 15% each with 0.25 sucrose)[13], (**15**%)[16], (**5**, 10, 15%)[26], (5, 10, 15%)[25], (not reported)[19]
Methanol	(5, 10, 15%)[25], (not reported)[19]
Trehalose	(**0.45 M**)[23]
Glycine	(5, 10, 20%)[4]
Addition method	Single step[1,2, 4, 5, 6, 7, 8. 10, 13, 16, 19, 20, 21, 25, 26], step-wise addition[19, 23]
Equilibration temperature (duration)	0 °C (20 min)[5], (10-30 min)[19]
	On ice (< 30 min)[10]
	0-4 °C (20 min)[21]
	4 °C (5 min)[22], 5 °C (10-30 min)[26]
	10 °C (10-30 min)[19]
	20 °C (10-30 min)[19], 21 °C (20 min)[13, 16]
	25 °C (< 15 min)[11], 26 °C (10, 30 min)[25]
	Not reported (**0**, 5, 10min)[7], (**3**, 30, 60 min)[8], (3, 15 min)[9], (~45 min)[23]
Time between collection and freezing	10 min[22], 30 min[10], < 1.5 h[4], 4 h[19]

Numbers in superscripts indicate the reference number assigned in Table 1.

Numbers in bold identify optimized protocols of that study.

Appendix. Continued.

8. Freezing container	Ampoules[4]: (1-mL)[1] (2-ml)[2]
	Plastic straw: (0.25-ml)[5, 6, 19, 23], (0.35-ml)[7], (0.5-ml)[8, 9, 11, 12, 19, 26], (2.5-ml)[19] (5-ml)[13, 16]
	Cryovials/cryotubes: (1-ml)[20], (1.5-ml)[22], (1.8-ml)[10], (2-ml)[3, 25], (4.5-ml)[23],
9. Freezing method	
Liquid nitrogen vapor : distance between sample and liquid nitrogen (reported cooling rate)	2 min[1]
	(8, 10, 12, 15, **17**, 20 cm above) x (2, **6**, 10, 14, 20, 30 min)[20]
	3 cm above for10 min (13.5 °C/min)[23]
	5 cm above: (3 min)[7], 10 min (114.3 °C/min)[12], with exposed straws (**79.8** °C/min to -60 °C)[12], with straws in sheath (38.2 °C/min to -60 °C)[12]
	7 cm above (8.4 °C/min at -20 °C)[9]
	10 cm above with exposed straws (57.5 °C/min to -60 °C)[12]
	From 0 to -80 °C at 5-13.5 °C/min, then to liquid nitrogen[5]
	From RT to -30 °C at 15 °C/min, then to liquid nitrogen[22]
	4.7 °C/min to -70 °C, then to liquid nitrogen[10]
Directly to liquid nitrogen	(Not reported)[3], (106.8 °C/min)[23]
Methanol/dry ice bath	60 min (9.4 °C/min)[12], 10 min (26.8 °C/min)[23]
Graybill and Horton method (1969):	(5, 30 °C/min)[4]
Controlled-rate freezer	Linde Model BF-4 Freezing chamber and controller[2, 5]: (1 °C/min to -8°C, -8°C to -25°C at 5.5°C/min, then to liquid nitrogen)[2]
	Planer Kryo 10 Mk II: (25 °C to -120 °C at 100 °C/min; at 15 °C/min to -150 °C, hold 1 min then to liquid nitrogen)[11], (15 °C to -30 °C at 2.5 °C/min; hold 5 min then to liquid nitrogen)[13, 16], (1, 5, 20, **50** °C/min)[19], (0 °C to -80 °C at 50 °C/min; held 10 min then to liquid nitrogen)[23], (9.5 °C/min)[23]
Deep freezer (-80 °C)	60 min (-6.1 °C/min)[12]
Commercial dairy freezing method	8 min run[26]
Not specified	(**6**, 11, 16, 21 °C/min to -70 °C, then to liquid nitrogen)[25]
10. Storage temperature (time)	-196 °C: (90 d)[2], (5 min – 68 d)[5], (< 3 d)[7], (1-2 d)[8], (30-80 d)[9], (1h - 4 yr)[12], (14 d - 30 d)[13], (7d)[16], (2 h – 1 d)[20], (1 h)[23], (2 d)[26]
	-190 °C: (7, 12, 30, 168, 217 d)[10]
	-170 °C: (24 h)[4]
	-5, -20, -40, -80 °C (< 2 min)[5]

Numbers in superscripts indicate the reference number assigned in Table 1.

Numbers in bold identify optimized protocols of that study.

Appendix. Continued.

11. Thawing	
Water bath	4 °C (3 min)[4]
	16-17 °C running water[20],
	16-17 °C running water, then move to 0–4 °C (10-14 min)[20]
	Room temperature (not reported)[8, 9], (> 1min)[12]
	20 °C (1 min)[7], (not reported)[11], (15 s)[19], (15-20 s)[23], (5-8 min)[23]
	22 °C (2 min)[4], 25 °C (30 s)[13], 40 °C (7 s)[26],
	48 °C (10 s)[5], 55 °C (20 s)[10], **60 °C (10 s)**[5], **70 °C (15 s)**[13, 16], (1 min)[22], 75 °C (2 s)[19]
Air bath	21 °C[2], 22 °C(5 min)[4]
Not specified	74 °C/min up to 26 °C[25]
12. Removal of CPA	Diluted in SW[4, 11, 16], Diluted in C-F HBSS[13], Step-wise removal[19, 23]
13. Post-thaw sperm quality assay	
Motility	Percentage[2, 9, 11, 13, 20, 26]
	Scale: (0-5)[7, 10], (1-4)[12], (0-4 ≈ 0-75%)[22], (0-5 at an increment of 0.5)[25]
Morphology	Cytogenetic examination[2], Scanning electron microscopy[9, 21]
Viability/survival	Eosin-nigrosine[9], Dye exclusion (0.3% trypan blue)[12], Comet assay[22]
Fertility	Absolute[1, 2, 4, 5, 6, 7, 8, 10, 12, 22, 23, 26], Relative to controls[7, 8]
	Polar body formation or appearance of first cleavage furrow[11]
	Count embryos at (1.5 h)[8, 25], (2-3 h)[1, 5, 6, 12, 26], (4 h)[7], (24 h)[22], (4-cell stage)[23]
	Subtraction of unfertilized eggs 6 h post-fertilization[4]
	Pooling abnormal embryos and normal D-larvae at 24 h[10]
Hatch	Absolute[1, 4, 13, 25, 26,], Relative to controls[12]
	Count straight-hinge larvae after (6 h)[12], (12 h)[13], (24 h)[4, 10, 26], (40 h)[1] (48 h)[25]
Larval growth	(2, 11 d)[5], (16 d)[10], (6 d)[12], (> 4 month)[16], (10 mm spat)[19], (metamorphosed spat)[23]
14. Fertilization method	
Methods	34 mL sperm to 5-15 million eggs in 250 mL SW[2]
	0.25 mL sperm to 30-300 ml SW with 200-900 eggs/mL[5]
	0.35 mL sperm to 1 mL of ova[7]
	0.5 mL sperm to 2000 eggs in 200 mL SW[10]
	0.5 mL sperm to 14,000 eggs in 500-mL plastic beaker[13]
	5 mL sperm to 2000 -12000 eggs in 200 mL SW[26],
	12-well tissue culture plates (30 µl sperm at 10^4- 10^7 cells/mL to 600 eggs in 3 mL SW of each well)[19, 23]
Eggs pooled or not	Pooled[4, 5, 7, 13, 16, 22, 23, 26] , Not pooled[1, 5, 13,
Sperm-to-egg ratio	(7300)[4], (10^4-10^5)[8], (10^3)[12], (~18000)[13], (10, 10^2, 10^3, **10^4,** 10^5, 10^6)[22], (10^2-10^5)[19, 23],
Control treatments	Positive control[1, 2, 4, 5, 7, 8, 10, 12, 13, 19, 20, 22, 23, 25, 26], Negative control[1, 5]
	Initial sperm quality control[4, 5, 23], Initial egg quality control[5]
	Toxicity control for fresh sperm[1], Toxicity control for fresh egg[4]

Numbers in superscripts indicate the reference number assigned in Table 1.

Numbers in bold identify optimized protocols of that study.

Cryopreservation of Sperm and Larvae of the Eastern Oyster

Carmen G. Paniagua-Chávez, John T. Buchanan, John E. Supan and Terrence R. Tiersch

Introduction

In aquaculture, cryopreservation studies have primarily addressed spermatozoa of four groups of commercially important fishes (catfishes, salmonids, tilapias and carps) (Stoss 1983, Chao and Liao 2001). However, work with larvae of fish or other organisms such as molluscs is scarce (Gwo 1995, Chao and Liao 2001). In molluscs, the Japanese oyster *Crassotrea gigas* has been the species most used to study cryopreservation of gametes and larvae (Table 1). Other species of oysters, including those of commercial importance, have not been studied. Cryopreservation of oyster gametes and larvae has been tested only in the laboratory (Paniagua-Chavez and Tiersch 2001, Ieropoli et al. 2004, Nascimento et al. 2005), and has yielded few reports of oyster growth beyond planktonic stages (Paniagua-Chavez et al. 1998a, Adams et al. 2004, Tervit et al. 2005). Given the benefit that this technique offers to research and the commercial oyster industry, cryopreservation of oyster gametes and larvae should be developed for application in the hatchery.

In the United States the most important oyster is the eastern oyster *Crassostrea virginica*. In Louisiana the production reached a farm value of ~ $ 30,000.00 in 2007 (Lutz 2007). Along the Atlantic and Gulf coasts, production of eastern oysters has declined due to a variety of reasons including a lack of consistent seed supply, excessive harvest, disease and natural predation (Supan and Wilson 1993). The production of cryopreserved gametes or larvae would improve hatchery production of seedstock oysters, thus allowing distribution of improved or genetically modified stocks.

This study represents the first successful production of seedstock from cryopreserved larvae of any aquatic food organism cultured for human consumption (Paniagua-Chavez et al. 1998a) and it documents for the first time the production of eastern oyster seedstock from eggs fertilized with thawed sperm.

Materials and Methods

Gamete Collection and Larvae Production

Collection of high quality gametes was necessary for reliable production of eastern oyster larvae suitable for cryopreservation. *Crassostrea virginica* can tolerate a wide range of salinities (osmolalities) in the natural environment. In the laboratory, osmolality must be controlled for gametes and larvae to be used for cryopreservation (Figure 1). Oysters from their natural environments were held in recirculating systems in the laboratory for at least 5 d at 18 to 20 °C. This temperature inhibited the Gulf coast oysters from spawning, but allowed maintenance of ripe gametes when the oysters were fed the marine microalgae *Isochrysis galbana* (T-iso) and *Chaetoceros calcitrans*. This acclimation period also allowed oysters that were stressed or weakened in transport to recover or die (reducing bacterial contamination of gametes and larvae). A marked

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Table 1. Relevant cryopreservation studies of gametes and larvae of oyster species.

Species	Frozen material	Citation
Crassostrea gigas	Sperm	Bougrier and Rabenomanana 1986
C. tulipa, *C. iredalei* and *C. gigas*,	Sperm	Yankson and Moyse 1991
Saccostrea cucullata	Sperm	Yankson and Moyse 1991
C. rhizophorae	Gametes and larvae	Nascimento et al. 2005
Pinctada fucata martensii	Sperm	Kawamoto et al. 2007
C. gigas	Sperm	McFadzen 1995
C. gigas	Sperm	Adams et al. 2004
C. gigas	Sperm	Dong et al. 2005
C. gigas	Sperm	Dong et al. 2007
C. gigas	Larvae	Renard and Cochanrd 1989
C. gigas	Larvae	Renard 1991
C. gigas	Larvae	McFadzen 1993
C. gigas	Larvae	Lin et al. 1993
C. gigas	Larvae	Chao et al. 1994
C. gigas	Larvae	Lin et al. 1994
C. gigas	Larvae	Gwo 1995
C. gigas	Larvae	Chao et al. 1997
C. gigas	Larvae and eggs	Naidenko 1997
C. gigas	Larvae	Usuki et al. 2002
C. gigas	Eggs	Tervit et al. 2005
C. virginica	Sperm	Huges 1973
C. virginica	Sperm	Zell et al. 1979
C. virginica	Larvae	Paniagua-Chavez et al. 1998a
C. virginica	Sperm and larvae	Paniagua-Chavez and Tiersch 2001

increase in production of larvae was noted after acclimation, and in some cases larvae could not be produced until after 5 d of acclimation. Oysters were held for 2 mo at 18 to 20 °C without spawning while allowing gamete maturation and retention (Buchanan et al. 1998).

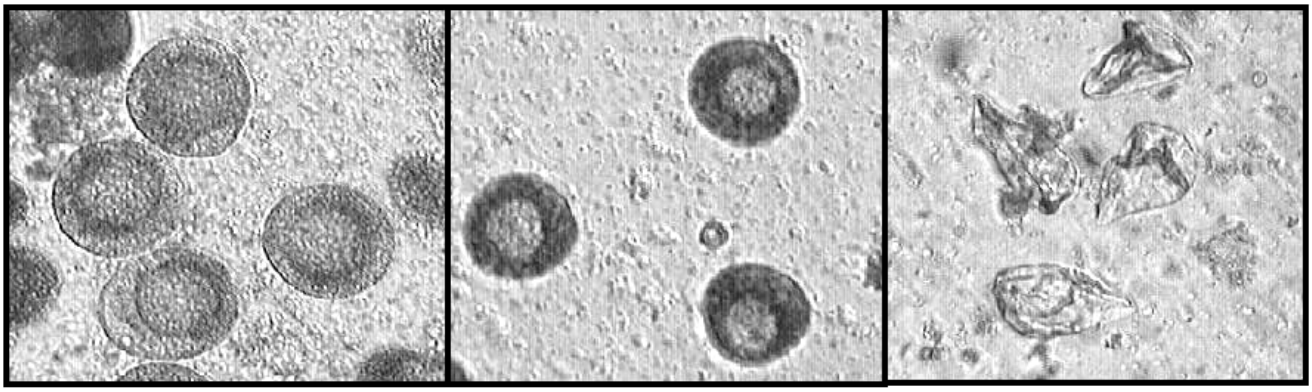

Figure 1. Eggs of *Crassostrea virginica* exposed for 5 min to artificial seawater (ASW) of various osmolalities. Prior to exposure, eggs were held at laboratory osmolalities (475 mOsmol/Kg) for 5 d. Left panel: eggs placed in hypotonic ASW at 115 mOsmol/Kg; middle panel: eggs placed in isotonic ASW at 468 mOsmol/Kg; right panel: eggs placed in hypertonic ASW at 705 mOsmol/Kg.

Before gamete collection, acclimated oysters were scrubbed with a mild bleach solution (5%) or were washed with 70% ethanol. Oysters were opened and inspected visually for ripeness, indicated by a creamy color with prominent genital canals running throughout the gonad. Mature oysters were selected and rinsed with 70% ethanol, followed by rinsing with filtered artificial seawater (ASW). To determine sex of the oysters, a sample from each gonad was taken by puncture with a capillary tube. This sample was smeared onto a glass slide and observed at 100-X magnification with phase-contrast microscopy. The gonads were lacerated with a sterile scalpel and the gametes were removed with a glass Pasteur pipette and placed into clean plastic beakers. All egg samples were passed through a 70-µm Nitex screen and were collected on a 13-µm screen. Eggs were washed with ASW and collected in a clean beaker. For sperm, samples were washed with calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez et al. 1998b) through 70-µm and 13-µm screens, and were re-suspended in C-F HBSS. Only actively swimming sperm were considered to be motile, and only males with sperm motility greater than 90% were selected for experimentation. To produce larvae, eggs were mixed with ~ 500 sperm per egg in 1 L of ASW. Embryos were cultivated at a concentration of ~ 100 embryos/mL in ASW. Trochophore larvae were collected ~ 12 hr after fertilization (incubated at 21 °C) on a 13-µm screen. Osmolality of all solutions was ~ 475 mOsmol/Kg. A 0.45-µm filter was used to sterilize the CF-HBSS. The ASW was passed through a 1-µm filter, sterilized with ultraviolet irradiation and passed through an activated carbon filter before use.

Cryopreservation Protocol

A single protocol was used to cryopreserve sperm and larvae (described below). Trochophore larvae were frozen at a concentration of 10,000/mL and sperm were frozen at ~ 1 x 10^9 mL. After suspension of sperm in C-F HBSS, or trochophore larvae in ASW, they were placed in a cryoprotectant solution composed of ASW and 15% propylene glycol (Sigma

Chemical Corp. St. Louis, Missouri). Five-mL straws were filled with the sperm or larvae suspensions and allowed to equilibrate for 20 min at 21 °C. The straws were frozen in a controlled-rate freezer (Kryo 10 series II, Planer, England). The cooling rate was -2.5 °C per min until reaching a final temperature of -30 °C, which was held for 5 min. Straws were plunged into LN_2 and stored for one week. A water bath was used to thaw the straws at 70 °C for 15 sec.

Hatchery Experiments

Samples were transported in nitrogen vapor shipping dewars (model CP-65, Taylor Wharton, Theodore, Alabama). In the hatchery, the straws were thawed in a water bath at 70 °C for 15 sec and drained into 1-L plastic beakers containing an equal volume of fresh filtered seawater. Thawed sperm was used to fertilize fresh eggs. A 30-µm mesh screen was used to remove thawed larvae from the water with cryoprotectant. Larvae collected on the screen were ready to culture in tanks.

Experimental Safeguards

Working at a hatchery on the coast created benefits and problems. We benefited from the availability of natural seawater, ambient conditions and natural (heterogeneous) food sources for grow-out of oysters after settlement. On the other hand, we needed to ensure that no extraneous natural larvae contaminated the experiment before settlement, and that natural spatfall did not influence our results during growth.

The procedures we employed to ensure that no natural larvae contaminated the experiment were: 1) working during late September which is at the end of the spawning season in Louisiana; 2) all oyster and algae tanks were segregated from sources of natural water and other sources of contamination; 3) all equipment and mesh screens were used exclusively for this experiment and were washed with filtered water before and after use; 4) all water used for rearing of oyster larvae and for culture of algae was filtered repeatedly through mesh sizes down to 1 µm; 5) the embryos and larvae in the experiments were collected on appropriate-sized mesh screens every 2 d, to perform water exchanges and counts; 6) experimental larvae were counted at each handling; 7) the larvae were also monitored to ensure that the populations were of the correct developmental stage; 8) counts of eyed pediveligers were performed as they were collected to be moved to the tanks containing cultch; 9) the number of larvae collected for settlement was compared with the larval census at that developmental stage, and 10) two additional mesh bags containing clean cultch were suspended among the experimental bags to estimate the occurrence of natural spatfall.

Results

The experiments were performed in two consecutive yr starting on September 18, 1996 and September 20, 1997 (Table 2). In the first yr, the experiment was terminated after 10 d due to a hurricane and flooding on the experiment site (Grand Isle, Louisiana). After 24 hr of incubation, 10% of the control larvae had developed to D-stage. Subsequently, survival of the control larvae decreased rapidly and after 10 d larvae were not found. Two percent of thawed larvae developed to D-stage and after 10 d of incubation 1,000 pediveligers were counted. Eggs fertilized with thawed sperm yielded better survival. After 24 hr, 1.1×10^6 D-stage larvae were counted, and after 10 d of incubation, 2.4×10^4 pediveligers were found.

In the s yr, larvae were incubated in 200-L tanks for 10 d, fed daily with 20 L of the algae *I. galbana* and were reared using normal hatchery procedures. After 24 hr of incubation, 28% of the control larvae, 24% of thawed larvae and 88% of larvae produced with thawed sperm had developed to D-stage. Larval numbers decreased throughout development, indicating absence of contamination with wild larvae. Larvae were collected by screening daily on 210-µm mesh beginning after 8 d of incubation and were transferred to a system containing cleaned shells of the common clam *Rangia cuneata* used as cultch material. A total of 500,000 control larvae, 28,000 thawed larvae and 64,000 larvae produced with thawed sperm were placed in the cultch system. After settlement, spat were secured in plastic bags and suspended in Caminada Bay (29º 15' 12" N, 90º 03' 26" W) to evaluate survival and growth. After 2 wk of suspension in the Bay, a subsample of 80 *R. cuneata* shells were obtained at random from each bag to count spat. One thousand spat were counted from the control group, 200 spat from the thawed larvae, 80 from larvae produced with thawed sperm and none from clean shells available for wild spat. After 4 mo of suspension in the Bay, all of the cultch material was examined and a total of 1,000 seed oysters (2.5 to 5 cm) were counted from the control group, 850 were counted from the thawed larvae and 230 from larvae produced with thawed sperm. At this time, a total of 57 wild spat were collected from the bags containing clean cultch (23 on one bag and 34 on the other).

Table 2. Number and percent of survival beyond settlement of Eastern oysters *Crassostrea virginica* produced from control larvae, thawed larvae and larvae produced with thawed sperm during 2 yr of research.

1996					
Date	**Control larvae**	**Thawed Larvae**	**Survival (% control)**	**Thawed Sperm**	**Survival (% control)**
9/18	3.0×10^6	3.0×10^6	100%	3.0×10^6	100%
9/20	3.1×10^5	5.0×10^3	2%	1.1×10^6	354%
9/22	3.8×10^4	3.6×10^3	9%	2.8×10^5	737%
9/24	6.0×10^3	2.0×10^3	33%	7.9×10^4	1,316%
9/26	4.6×10^3	2.0×10^3	43%	3.4×10^4	739%
9/30*	0	1.0×10^3		2.4×10^4	

Table 2. Continued.

	1997				
Date	**Control Larvae**	**Thawed larvae**	**Survival (% control)**	**Thawed Sperm**	**Survival (% control)**
9/20	6.0×10^6	3.0×10^6	50%	3.0×10^6	50%
9/22	1.7×10^6	4.0×10^5	24%	1.5×10^6	88%
9/24	1.0×10^6	2.0×10^5	20%	6.3×10^5	63%
9/26	1.0×10^6	5.3×10^4	5%	5.0×10^5	50%
9/28	5.6×10^5	2.8×10^4	5%	6.4×10^4	11%
Set on cultch					
10/97**	1.0×10^3	2.0×10^2	20%	8.0×10^2	8%
1/98***	1.0×10^3	8.5×10^2	85%	2.3×10^2	23%

*Experiment terminated due to hurricane.
**Total number of spat counted on a subsample of 80 *R. cuneata* shell (cultch). No natural spatfall was observed on any cultch samples on this date.
***Natural spatfall was observed only on clean cultch (not in control or thawed treatments). Oysters from wild spatfall were visibly smaller than those from the experimental groups.

The wild spat were less than 0.5 to 1 cm in length, while the experimental spat (from control, thawed larvae and sperm) were more than 2.5 cm at this time, allowing identification of naturally produced spat. The combination of few natural spat at this time of yr and a greater suitability for settlement on the clean cultch may explain the lack of wild spat in the experimental bags. During the experiments, all larvae and spat appeared to be morphologically normal and healthy (Figure 2).

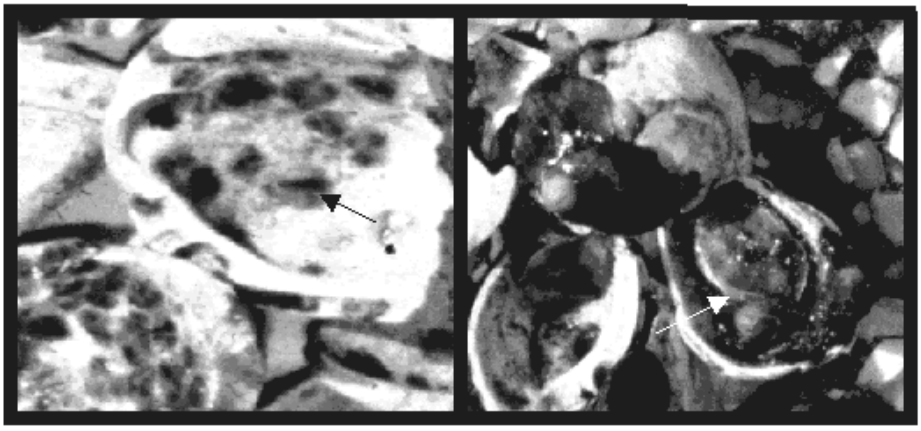

Figure 2. Eastern oysters produced from thawed larvae. Left: spat 2 wk after settlement on shell of *Rangia cuneata* clam. Right: Oysters 4 months after settlement.

Discussion

The goal of applied research is the transfer of technology from the laboratory to the industrial or commercial level. In aquaculture, technology transfer involves modification of laboratory techniques for use in hatcheries or other culture environments. Cryopreservation of gametes and embryos is a good example of technology that can be used in aquaculture. However, most of the studies on cryopreservation of oyster sperm and embryos have been limited to a few d in the laboratory and have not been evaluated for scaling up for production. It is important to evaluate laboratory studies because often they do not yield the same results as those performed in the hatchery or natural environment. The growth of thawed larvae and larvae produced from eggs fertilized with thawed sperm for a few hr or d does not ensure survival through settlement or production of adult oysters. Genetic or physiological damage could result in production of abnormal larvae, excessive mortality, or the inability to complete metamorphosis and attach to cultch.

In our study, we found that it was possible to obtain settlement and to produce normal seed oysters from thawed trochophore larvae and eggs fertilized with thawed sperm. Survival, although apparently low, was not different from the control group, and was not different from normal survival in the hatchery. Extremely large numbers of larvae (in the billions) are routinely used to produce seedstock at the commercial level. With additional research, cryopreservation could be scaled up to allow production of commercially relevant numbers of larvae.

This is the first successful production of seedstock from cryopreserved larvae of any species of aquatic organism commercially grown for human consumption. This is also the first production of seed oysters from eggs fertilized with thawed sperm. The production of oysters capable of being planted on oyster grounds for growth and harvest demonstrates that cryopreservation can be applied for use in the hatchery. This is especially important for molluscs because at present maintenance of broodstock requires considerable space and expense within a hatchery, or entails the risk of placing valuable stocks into natural waters where they are susceptible to diseases, predators and contamination with wild spatfall.

The availability of frozen larvae and sperm offers obvious benefits. The frozen material would ensure protection of valuable stocks and would facilitate transport and availability of improved (e.g. disease-resistant) lines. The management of genetic resources of endangered species (Tiersch et al. 1998), and the techniques for larval cryopreservation may be applicable to highly endangered molluscs, such as freshwater mussels. Research efforts would benefit from standardization (over distance and time) due to the availability through cryopreservation of control lines of oysters and economical storage of specific research populations (e.g. tetraploid larvae). Work could proceed yr-round given the availability of larvae outside of the spawning season. This point has special relevance to cytogenetic studies of oysters given that transformed cell lines do not exist, and larval material is often used to yield primary cultures with sufficient mitotic activity to reliably produce metaphase chromosomes (Zhang et al., this volume). However, future studies should evaluate the complete life cycle of oysters produced from cryopreserved larvae or sperm, and address optimization of production.

Over the last 20 yr, the production of Japanese oyster seed in hatcheries has been economically viable in Washington state (Lipovsky 1980). However, for the eastern oyster, economic feasibility studies of hatcheries in the Chesapeake Bay have shown that hatcheries operating solely as seed oyster facilities, rather than as a component of a harvesting operation, have the ability to generate profit. However, this potential relies heavily on favorable growth

conditions in the hatchery. Salinity fluctuations or equipment failure could drastically reduce the hatchery output (Lipschultz and Krantz 1980). A good option to optimize production is to use cryopreserved sperm and larvae when favorable growth conditions are found in the hatchery. A cost analysis has been performed for the integration of cryopreservation into existing fish hatcheries (Caffey and Tiersch 2000, and Caffey and Tiersch, this volume). Given the possibility of using cryopreserved oyster larvae and sperm in the hatchery, a similar analysis should be performed for the application of cryopreservation to oyster production.

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Cryopreservation of Eggs and Embryos of Shellfish

Ta-Te Lin and Nai-Hsien Chao

Overview

Cryopreservation of shellfish embryos allows for gene bank establishment and manipulation of spawning programs. Embryos of three species were studied to establish cryopreservation protocols in this research: Pacific oyster *Crassostrea gigas*, hard clam *Meretrix lusoria* and small abalone *Haliotis diversicolor diversicolor*. As a prerequisite step to develop practical cryopreservation procedures, the osmometric characteristics, intracellular ice formation, and cryoprotectant toxicity tolerance of eggs and embryos to various cryoprotectants were investigated. Based on this fundamental information, a conventional two-step freezing protocol to cryopreserve Pacific oyster and hard clam embryos was established and optimized. For Pacific oysters, late morula stage embryos (4 hr at 28 °C after artificial fertilization) were equilibrated in 2 M dimethyl sulfoxide (DMSO) plus seawater for 10 min at 25 °C and were cooled at -1 °C per min from 0 to -12 °C. Straws containing embryos were held at -12 °C for 5 min allowing equilibration after seeding. The embryos were slowly cooled at -2 °C per min to -35 °C and allowed 5 min for equilibration before plunging in LN_2. After rapid warming in a water bath at 28 °C, embryos were placed in seawater to unload DMSO. The survival rate of cryopreserved Pacific oyster embryos was 78 ± 8% using this protocol. For hard clam embryos, a survival rate of 72 ± 10% was achieved using the same freezing protocol. However, this approach could not be applied successfully to embryos of the small abalone. With freezing rates ranging from -0.25 to -2 °C per min, few small abalone embryos survived below -30 °C. Using vitrification as an alternative approach to cryopreserve Pacific oyster embryos, survival of 14 ± 8% was obtained.

Introduction

Cryopreservation of shellfish embryos is of significant importance, because it allows gene bank establishment and manipulation of spawning programs. Although reports on the cryopreservation of shellfish embryos are not abundant, several cryopreservation techniques for shellfish embryos have recently been assayed using different cryoprotectants (CPAs) or freezing protocols (Toledo 1991, Lin et al. 1993a, Gwo 1995, Chao et al. 1997, Naidenko 1997). In order to improve protocols to result in higher survival of shellfish embryos following cryopreservation, their osmometric characteristics, intracellular ice formation (IIF) (Pitt and Steponkus 1989) and toxicity tolerance to various CPAs were investigated. This information is useful in designing practical cryopreservation procedures and saves efforts in the process of optimizing procedures.

This chapter focuses on the introduction of necessary measurements and their implications on the cryobiology of selected shellfish embryos, and on the comparison of procedural factors affecting the survival of cryopreserved embryos. Three species of shellfish embryos were selected to establish cryopreservation protocols. They were the pacific oyster, hard clam and small abalone. The cryopreservation protocol was designed based on the information from fundamental measurements and the protocols were further optimized by modifying the CPA loading and unloading procedures, freezing rates, seeding temperatures and holding time.

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Methods

Preparation of Samples

Sperm and eggs were collected from male and female oysters cultured in seawater of 34 ± 2% salinity. Embryos were obtained after artificial fertilization and were cultured in seawater at 28 °C for 4 hr. At fertilization the number of sperm surrounding each egg was adjusted to 5 ± 1. Normally more than 90% fertilization was obtained from this procedure. In the case of hard clams, broodstock were placed in a basin with the temperature increased from room temperature to 30 °C at 1 °C per min; the water was allowed to return to room temperature with natural cooling. Spawning usually occurred following the drop in temperature and artificial fertilization was undertaken with a 5:1 ratio of sperm to eggs (Chao et al. 1997). For small abalone, after exposing the broodstock to air drying for 30 min, water temperature was raised 1 °C per hr for less than 4 hr and then quickly lowered to 2 °C below the starting temperature. One or two cycles of such temperature stimulation usually induced spawning.

Osmometric Measurements

The rational design of cryopreservation protocols requires characterization of static and dynamic osmometric behaviors. This information can be used to simulate the volumetric responses and the extent of supercooling of the embryos during a freezing and thawing process (Mazur 1963).

Hard clam and small abalone eggs were collected and transferred to a micro-diffusion chamber (Lin et al. 1989) to measure their volumetric change when subjected to a variation of osmotic environment at controlled temperature. Static measurements of the volume of individual eggs at different tonicities were conducted in a stepwise sequence of tonicity changes. Eggs were exposed to each solution for sufficient time to allow for full equilibration and the cellular volume was determined from the measured cross-sectional area assuming a spherical shape.

To determine the hydraulic conductivity, individual eggs were subjected to a change from isotonic seawater (992 mOsmol/Kg) to hypertonic seawater (2,000 mOsmol/Kg) at 0, 10, 20 or 30 °C. The volumetric data for each egg was measured from digitized images and recorded.

The permeability of different cryoprotectants such as ethylene glycol, glycerol, DMSO, acetamide and propylene glycol were compared by measuring the volumetric behavior of eggs (Lin et al. 1992, Lin et al. 1993b).

Intracellular Ice Formation (IIF) Experiments

During rapid cooling, IIF occurs over a broad range of subfreezing temperatures in a diverse array of biological cells (Mazur 1977, Steponkus 1984). Characterization of the temperature distribution at which IIF occurs is of primary importance in predicting the probability of IIF during freezing (Pitt et al. 1991).

Oyster eggs or embryos were transferred to the glass slide of a directional cryomicroscope (Lung and Lin 1994) for the measurement of IIF temperature when subjected to various subfreezing temperatures. The experiments were recorded with a video recorder and the temperature at which ‘flashing’ (darkening) of eggs or embryos occurred was determined from the video tape during later analysis (Steponkus et al. 1984). Experiments were conducted for Pacific oyster eggs and embryos. The samples were cooled to the desired subfreezing temperature at a cooling rate of -60 °C per min and held at constant temperature to record the

time when the IIF occurred. Approximately 50 samples were recorded for each experiment and cumulative frequency of IIF was plotted.

Experiments on the Toxicity Tolerance to Cryoprotectants

The apparent toxicity of CPAs is dependent on the type and concentration of CPA, the equilibration time and temperature during loading. The stage of embryonic development is also a critical factor in various biological systems (Weber and Youngs 1994). In experiments on the toxicity tolerance of oyster embryos to selected CPAs, embryos of different development stages from 60 to 240 min after artificial fertilization in 27 ± 1 °C seawater were tested. Four conventional CPAs, acetamide (A), DMSO, ethylene glycol (EG) and propylene glycol (PG), with concentrations of from 1 to 5 M were used (Chao et al. 1994).

Cryopreservation Experiments

For use of a conventional two-step freezing method, preliminary experiments were performed in a programmable alcohol bath with maximum cooling capacity of 2 °C per min and a minimum temperature of -65 °C. Experiments presented in this chapter were performed in a programmable freezer (KRYO 10 Series III, Planer Biomed, England).

For Pacific oysters, late morula stage embryos (4 hr at 28 °C after artificial fertilization) were equilibrated in 2 M DMSO (or different CPAs) plus seawater for 10 min at 25 °C and were cooled at -1 °C per min from 0 to -12 °C (or different seeding temperatures). Straws (0.5 mL) containing embryos were held at -12 °C for 5 min allowing equilibration after seeding. Embryos were cooled at -2 °C per min (or various freezing rates) to -35 °C and held 5 min for equilibration before plunging in liquid nitrogen (LN_2). After warming in a water bath at 28 °C, embryos were placed in seawater to unload DMSO. The rate of survival was assayed by counting the embryos with active rotary motion.

Vitrification was also investigated as an alternative approach to cryopreserve the embryos of Pacific oyster and small abalone. Embryos were preloaded with 2 M DMSO for 20 min at 20 °C, 3 M DMSO plus 1 M ethylene glycol for 2 min at 0 °C, and were dehydrated in a solution containing 5 M DMSO, 3 M ethylene glycol and 6% polyvinyl pyrrolidine (PVP) for 1 min at 0 °C before plunging in LN_2. After thawing in 28 °C water bath, embryos were diluted with 3 M DMSO plus 1 M ethylene glycol plus 1 M sucrose for 2 min at 0 °C, and 2 M DMSO plus 0.3 M NaCl for 5 min at 20 °C, and finally transferred to seawater at 20 °C.

Results

Equilibrium Osmometric Behavior

The volumetric data from the static measurement of small abalone eggs, hard clam eggs and Pacific oyster embryos indicated that their behavior was nearly ideal over the range of 300 to 3,000 mOsmol/Kg (Figure 1). The fractional volume of 1.0 indicates the equilibrium volume at isotonic condition (992 mOsmol/Kg). The three measurements were similar and the average values of the non-osmotic fractional volumes were 0.49 for oyster embryos, 0.47 for hard clam eggs and 0.45 for small abalone eggs. These values were similar to one another, but were much larger than those of most mammalian and plant cells.

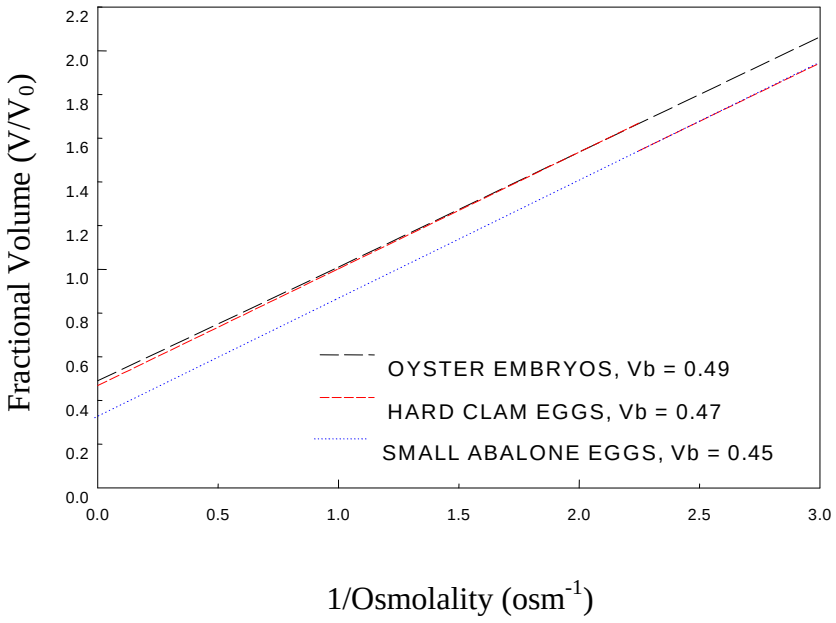

Figure 1. Comparison of Boyle van't Hoff relationship (Nobel 1969) of Pacific oyster embryos and eggs of hard clams and small abalone.

Non-equilibrium Osmometric Behavior

The hydraulic conductivities (L_p), of eggs of hard clam and small abalone were determined at various temperatures (Figure 2). The L_p values were determined from the volumetric data measured with the micro-diffusion chamber. The data of individual eggs were fitted with the classical mathematical model and the average values were obtained (Lin et al. 1989). The estimated L_p of hard eggs was $8.4 \pm 1.5 \times 10^{-10}$ m/(s-atm) at 0 °C, $12.7 \pm 3.6 \times 10^{-10}$ m/(s-atm) at 10 °C, $23.1 \pm 8.6 \times 10^{-10}$ m/(s-atm) at 20 °C and $36.9 \pm 20.7 \times 10^{-10}$ m/(s-atm) at 30 °C. Estimates of L_p among individual hard clam eggs were highly variable. Fitting an Arrhenius relation to the combined data yielded an activation energy of 7.5 ± 10.3 kcal/mole-K. Similarly, the L_p values of small abalone eggs were determined to be $3.65 \pm 2.00 \times 10^{-9}$ at 10 °C, $6.36 \pm 2.30 \times 10^{-9}$ at 20 °C, $12.89 \pm 3.44 \times 10^{-9}$ at 30 °C. The activation energy was 11.6 ± 0.6 kcal/mole-K which was larger than that of hard clam eggs indicating stronger temperature dependency. Estimated L_p of hard eggs was $8.4 \pm 1.5 \times 10^{-10}$ m/(s-atm) at 0 °C, $12.7 \pm 3.6 \times 10^{-10}$ m/(s-atm) at 10 °C, $23.1 \pm 8.6 \times 10^{-10}$ m/(s-atm) at 20 °C and $36.9 \pm 20.7 \times 10^{-10}$ m/(s-atm) at 30 °C. Estimates of L_p among individual hard clam eggs were highly variable. Fitting an Arrhenius relation to the combined data yielded an activation energy of 7.5 ± 10.3 kcal/mole-K. Similarly, the L_p values of small abalone eggs were determined to be $3.65 \pm 2.00 \times 10^{-9}$ at 10 °C, $6.36 \pm 2.30 \times 10^{-9}$ at 20 °C, $12.89 \pm 3.44 \times 10^{-9}$ at 30 °C. The activation energy was 11.6 ± 0.6 kcal/mole-K which was larger than that of hard clam eggs indicating stronger temperature dependency.

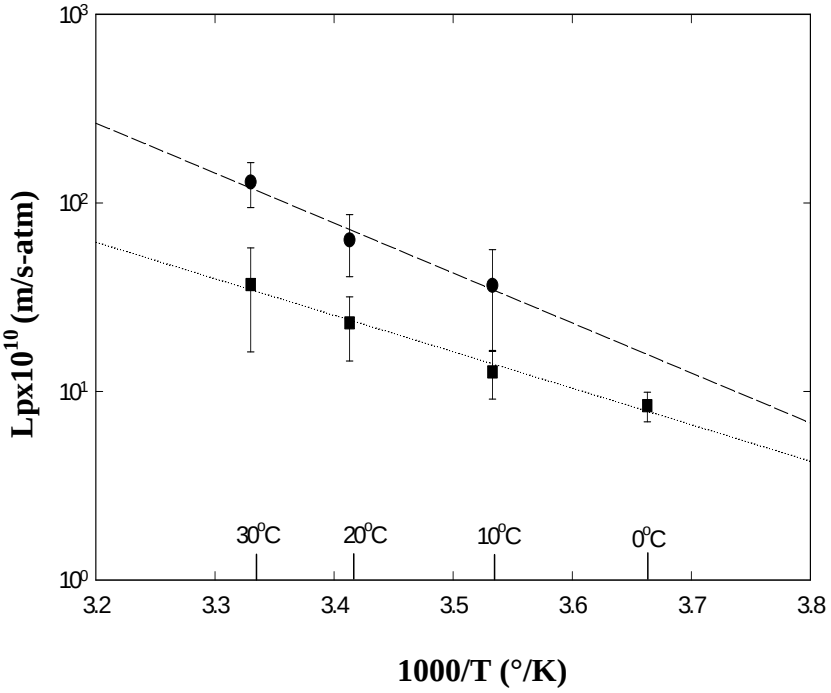

Figure 2. Comparison of hydraulic conductivity and its temperature dependence of small abalone eggs (circles) and hard clam eggs (squares).

Cryoprotectant Permeation

As an example, Figure 3 compares the volumetric excursions of small abalone eggs subjected to permeation of ethylene glycol, acetamide, propylene glycol and glycerol at the temperature of 20 °C and at the concentration of 2 M. The shrink-swell curve for permeation of different CPA revealed different rates. The permeation rate of DMSO (not shown), propylene glycol and acetamide appeared to be similar and to reach equilibration in about 20 min. The permeation of ethylene glycol into small abalone eggs was slower and reached a 90% equilibration in about 30 min. Glycerol did not permeate small abalone eggs judging from their constant volume after the shrinkage phase of the volumetric excursion. A parallel study on the CPA permeation to hard clam eggs was also carried out and the results were similar (Lin et al. 1993b).

Intracellular Ice Formation

Darkening of eggs or embryos can be clearly observed on the directional cryomicroscope during freezing. Figures 4 and 5 show the cumulative frequency of IIF versus time during a subfreezing isothermal period for oyster eggs and embryos in seawater. It is clear that the occurrence of IIF is time and temperature dependent. The probability of IIF increased with time and lower temperature. Total incidence of IIF was less than 100% at warmer temperatures because dehydration of eggs or embryos occurred.

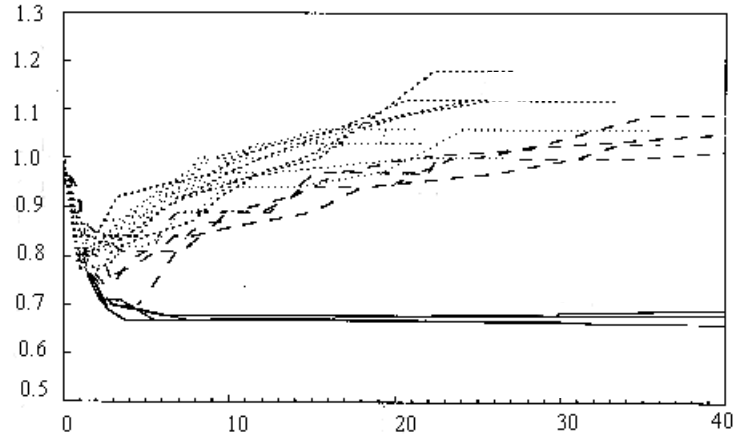

Figure 3. Volumetric excursion of small abalone eggs subjected to different types of cryoprotectants at a 2 M concentration during the isothermal period at 20 ºC.

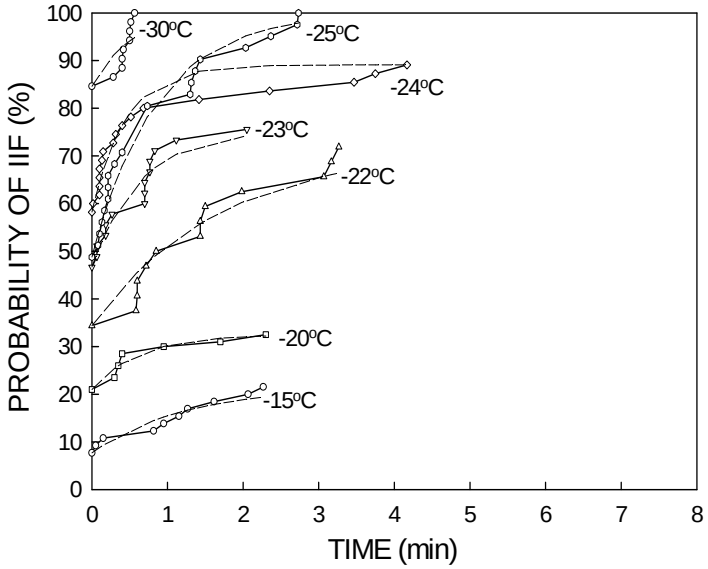

Figure 4. Cumulative frequency of intracellular ice formation (IIF) of Pacific oyster eggs at various subfreezing temperatures. Eggs were initially in seawater. The dashed lines were curves fitted with a stochastic model (Lin and Lung 1995).

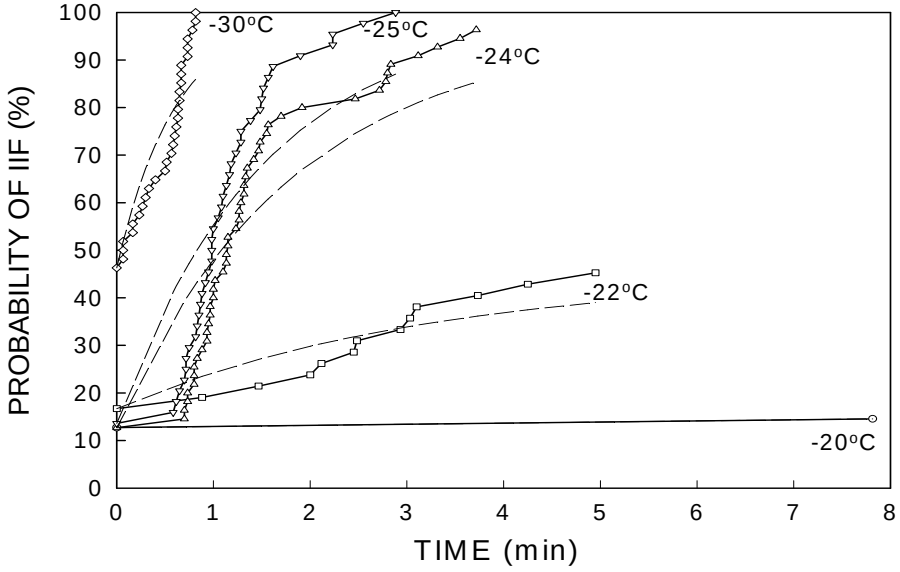

Figure 5. Cumulative frequency of intracellular ice formation (IIF) of Pacific oyster embryos at various subfreezing temperatures. Embryos were initially in seawater. The dashed lines were curves fitted with a stochastic model (Lin and Lung 1995).

The median IIF temperature of embryos and eggs were determined to be -22.5 °C for embryos and -21.0 °C for eggs (Lin and Lung 1995). The transition temperature zone of IIF probability from 0 to 1 appeared to be broader for embryos than for eggs.

Toxicity Tolerance

The apparent toxicity of cryoprotectants is dependent on the type and concentration of CPA, and the temperature and duration of exposure (Fahy 1984). To choose pertinent cryoprotectant for the cryopreservation of selected shellfish embryos, the toxicity tolerance to CPAs was investigated (Chao et al. 1994). As an example, Figure 6 depicts the effect of exposure of Pacific oyster embryos to different concentrations of DMSO plus seawater for 5 min at 27 ± 1 °C. The apparent toxicity of DMSO tended to increase as the concentration increased from 1 to 5 M. However, there was no significant difference in survival among the embryos exposed to less than 3 M DMSO. A notable decrease in survival occurred when concentrations of DMSO were above 4 M. Late stage embryos appeared to be more tolerant to CPA toxicity. For the four CPAs tested (on a molar-equivalent basis), DMSO appeared to be less toxic than propylene glycol, ethylene glycol and acetamide in general.

Conventional Two-step Cryopreservation Experiments

The survival of Pacific oyster embryos 4 hr after fertilization at consecutive steps during freezing at a rate of -2 °C per min with 2 M DMSO as CPA was studied (Figure 7). The reduction of survival was minimal at the CPA loading step. This was consistent with the results from the toxicity tolerance experiments. There was a decrease in survival during the freezing to -35 °C and the plunging process. With the optimized cryopreservation protocol described above, a

survival rate of 78 ± 8% was obtained for Pacific oyster embryos quenched in LN_2. Using the same protocol for hard clam embryos 5 hr after fertilization, the survival rate was 72 ± 10%. A survival rate of 13 ± 6% was obtained for Pacific oyster eggs at a freezing rate of -0.5 °C per min.

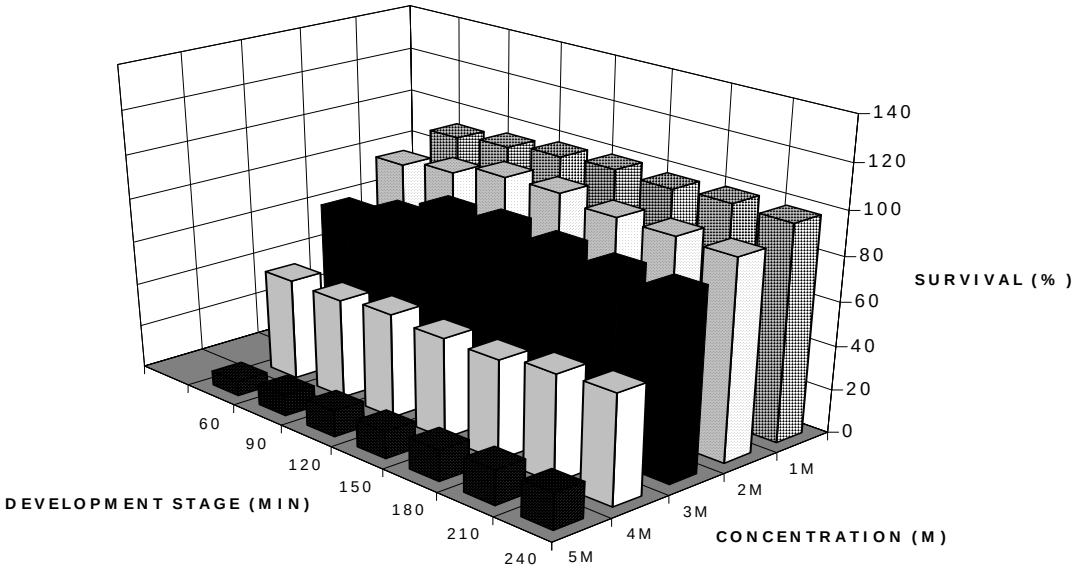

Figure 6. Toxicity tolerance of Pacific oyster embryos of various development stages to DMSO of various concentrations (Chao et al. 1994).

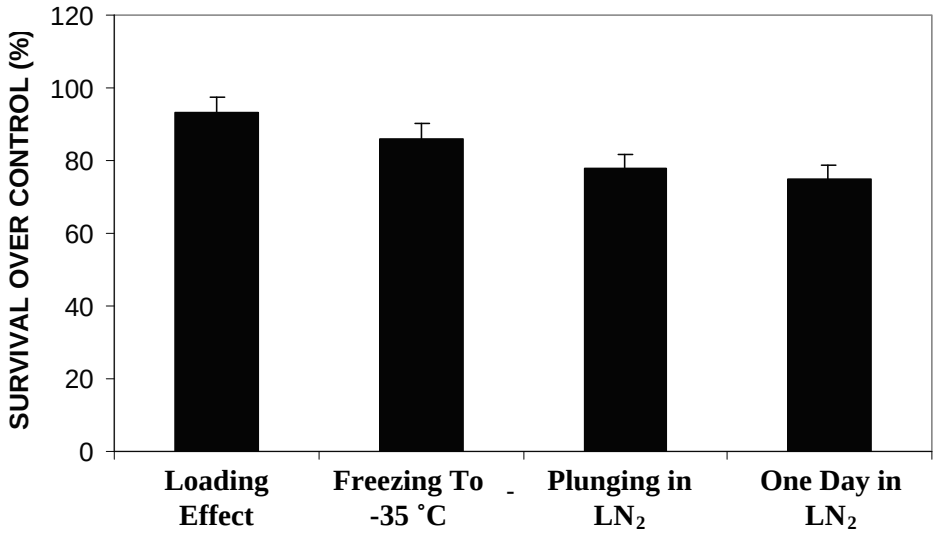

Figure 7. Survival of Pacific oyster embryos 4 hr after fertilization at consecutive steps during two-step freezing at a rate of 2 °C per min with 2M DMSO used as cryoprotectant.

In the process to optimize cryopreservation protocols, cooling rate was the most critical factor to be considered. The effect of freezing rate on the survival of Pacific oyster embryos loaded with 2 M DMSO or 2 M glycerol was studied (Figure 8). The optimum cooling rate appeared to be -2 °C per min for DMSO and -4 °C per min for glycerol. This result also indicated that various levels of survival of Pacific oyster embryos can be obtained over a relatively broad range of cooling rates.

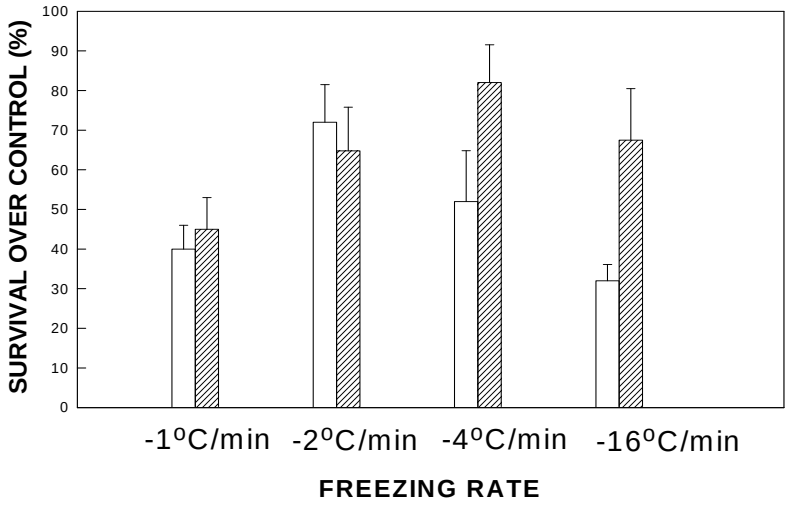

Figure 8. Effect of freezing rate on the survival of cryopreserved Pacific oyster embryos loaded with 2M DMSO (open bars) or 2M glycerol (hatched bars).

Comparisons of the effect of using DMSO, ethylene glycol and propylene glycol as CPA during freezing of Pacific oyster embryos were made (Figure 9). The freezing rate was -2 °C per min. In combination with the results above (Figure 8), there was no significant difference in using DMSO, ethylene glycol or glycerol as CPA for Pacific oyster embryo cryopreservation. However, there was a significant drop in survival rate using propylene glycol as CPA.

Before freezing with a constant cooling rate, manual seeding was necessary. This procedure was accomplished by gripping the straws with a pair of tweezers cooled in LN_2. The cold temperature induced the nucleation of the supercooled solution in the straw and the ice propagated until the solution was completely solidified. Seeding temperature was also examined with experiments in the protocol optimization process. In the range of from -10 to -14 °C, seeding at -12 °C yielded the best results. Seeding at warmer temperatures resulted in melting of the solid phase induced by the seeding procedure and the solution in the straw remained supercooled. Following seeding, or at the end of the constant-rate freezing (at -35 °C), holding at isothermal conditions for a period of time was beneficial to allow for intracellular and extracellular osmotic equilibration. However, experiments also revealed that longer holding times increased the probability of IIF and thus decreased the survival rate.

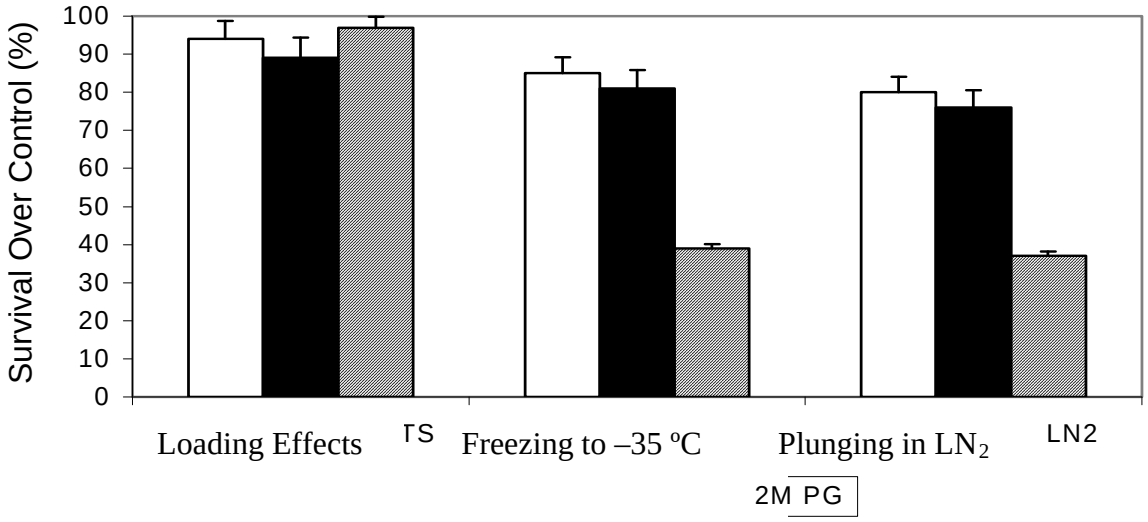

Figure 9. Comparisons of the effect of using dimethyl sulfoxide (white bars), ethylene glycol (black bars) or propylene glycol (grey bars) as cryoprotectants for Pacific oyster embryos.

Vitrification

Although the application of the conventional two-step method to embryo cryopreservation of Pacific oyster and hard clam was successful, the same approach could not be applied to embryos of small abalone. With freezing rates ranging from -0.25 to -2.0 °C per min, few small abalone embryos survived below -30 °C. Therefore, vitrification was considered as an alternative approach. Using a combination of DMSO and ethylene glycol and the vitrification procedure described above, we were able to obtain some surviving small abalone embryos (<1%). For Pacific oyster embryos, the survival rate was 14 ± 8%. A significant drop of survival rate during loading of high concentrations of CPA and plunging in LN_2 indicated that it will be possible to improve the vitrification procedure.

Conclusions

1. Fundamental measurement of osmometric characteristics, intracellular ice formation and toxicity tolerance aided in design and optimization of cryopreservation procedures for selected shellfish embryos and eggs.
2. The effect of cooling rate, type of CPA, seeding temperature and holding time were examined to optimize the two-step freezing protocol.
3. Survival rate about 75% were achieved for Pacific oyster and hard clam embryos after optimization of the freezing procedures.
4. Survival of embryos of small abalone was <1% and Pacific oyster embryos was 14 ± 8% using current vitrification procedures. Further optimization of the vitrification procedure is required.

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Annotated Bibliography of Developments in the Last Decade

Cryopreservation of shellfish embryos was realized before 2000, and cryopreservation of shellfish oocytes has been accomplished since then. This success offers the opportunity to preserve germplasm from more individuals within species or lines, thereby improving the genetic diversity of preserved stocks.

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New Chapter

Cryopreservation of Pacific Oyster Oocytes

Serean L. Adams, H. Robin Tervit, Liliana Salinas-Flores, John F. Smith, Lindsay T. McGowan, Rodney D. Roberts, Achim R. Janke, Nick G. King, Stephen C. Webb and Samantha L. Gale

Introduction

The Pacific oyster *Crassostrea gigas* is the most widely farmed aquaculture species worldwide (FAO 2008). In some countries, the animals spawn naturally and the juveniles (called "spat") are collected and then on-grown by farmers. However, in other countries natural spat-fall is unreliable or insufficient for production needs. In these countries, shellfish hatcheries produce spat for on-growing (Lapègue et al. 2007). Hatcheries can also carry out selective breeding to establish superior genetic lines or oysters with specific traits (e.g., disease resistance, high meat-to-shell ratio). Cryopreservation goes hand in hand with selective breeding as it provides greater control over parental crosses and reduces the cost of maintaining broodstock from a large number of family lines. Genetic material can be stored beyond the lifespan of the parents and a repeated supply of gametes can be obtained from specific individuals. Cryopreservation also manages the commercial and biological risks that are associated with selective breeding: for example, economic and market changes in desirable traits or animal loss due to disease. In hatcheries, cryopreservation of oocytes, sperm, or larvae would enable spat production year round without having to condition animals for out-of-season production.

In New Zealand, the Cawthron Institute is running a selective breeding program for Pacific oysters. Alongside this program, research is being carried out to develop robust cryopreservation methods for oocytes and larvae. Successful cryopreservation of Pacific oyster oocytes was first reported by Tervit et al. (2005). However, further work is needed to ensure that cryopreservation can be reliably applied to selective breeding. A method for cryopreserving Pacific oyster sperm has also been developed and this is now being applied in selective breeding (Smith et al. 2001, Adams et al. 2004, 2008a,b).

In contrast to the small number of oocytes available for cryopreservation in agricultural species, oysters, like other shellfish, produce millions of oocytes with each spawning. This means that hundreds of thousands of offspring can be produced from a given female and genetic gains can be made easily through maternal as well as paternal lines. Cryopreservation of oocytes of any species is not routine. Several features of oocytes make them less amenable to cryopreservation than other cell types. Firstly, oocytes are relatively large. They vary considerably in size but generally have a small surface area-to-volume ratio when compared with other cell types such as sperm. Those of the Pacific oyster are approximately 50 μm in diameter and have an osmotically inactive fraction of 0.48 (Salinas-Flores et al. 2008a). This means that the oocytes must lose more water (when compared with other cell types) to survive cryopreservation and that slow cooling rates must be used. Secondly, oocytes of many species are held at either metaphase I or metaphase II before being activated by sperm. The meiotic spindle and actin cytoskeleton present during these developmental stages are sensitive to lower temperatures and may undergo depolymerization during cooling (Magistrini and Szöllösi 1980,

Adams, S. L., H. R. Tervit, L. Salinas-Flores, J. F. Smith, L. T. McGowan, R. D. Roberts, A. Janke, N. G. King, S. C. Webb, and S. L. Gale. 2011. Cryopreservation of Pacific oyster oocytes. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 616-623.

Amann and Parkes 1994, Saunders and Parkes 1999, Songsasen et al. 2002). This often results in abnormal spindle formation and aneuploidy. Oocytes of the Pacific oyster are maintained in the gonad at prophase I but most spontaneously mature to metaphase I when they are stripped or spawned into seawater (Colas and Dubé 1998). Thirdly, oocytes are often sensitive to chilling injury (Arav et al. 1996, Pearl and Arav 2000), although this has not proven to be the case for Pacific oyster oocytes (Roberts et al. 2004, Tervit et al. 2005). In fact, these oocytes can be stored for several d at 5 ^{o}C and fertilize and develop normally.

This chapter summarizes research towards developing a robust method for cryopreserving Pacific oyster oocytes for use in selective breeding and hatcheries. Critical steps in the method are outlined as well as subsequent experiments carried to improve the method.

Methodology and Results

Gamete Collection, Fertilization, and Larval Development Assays

Mature gametes were obtained and fertilization assays carried out as described by Adams et al. (2004) and Tervit et al. (2005). Briefly, sperm and oocytes were recovered by physical stripping of the gonad. Sperm were stripped as "dry" as possible (i.e., in a minimal volume of seawater). Oocytes were washed and resettled in ~30 mL of 1-µm filtered seawater. Sperm and oocytes were stored separately at 5 °C until used. Fertilization assays were carried out in 12-well tissue culture dishes containing 3 mL of filtered seawater with oocytes at a concentration of 200/mL and sperm at a concentration of 10^{6}cells/mL. Duplicate assays were carried out for each treatment and pool. Development was halted by the addition of 10% formalin after cleavage (2-8 cells) and the percentage of oocytes fertilized was determined (n = ~100 per well). For larval development assays, 1 million oocytes were diluted to a density of ~1,000/mL and fertilized at a ratio of 100 sperm per oocyte. After a contact time of 10 to 20 min, the fertilized oocytes were transferred to tanks containing 150 L of filtered seawater and 1 mg/L ethylenediaminetetraacetic acid (EDTA) at ~23 ^{o}C. After 24 to 48 hr, the tanks were drained through a 45-µm mesh and samples were taken to determine the percentage of oocytes developing to D-stage larvae in each treatment.

Oocyte Cryopreservation

A range of factors such as cryoprotective agent (CPA) type and concentration, water type, holding temperature and cooling rate were evaluated in a series of experiments (Tervit et al. 2005) using fertilization assays to measure post-thaw viability. The CPAs were prepared at double their final concentration in Milli-Q water or seawater and added to the oocytes gradually in 10 fixed volume steps, 1 min apart to avoid osmotic shock. The oocytes were frozen in 0.25-mL straws using controlled-rate freezers programmed to hold at 0 ^{o}C for 5 min, cool at 1 ^{o}C/min to various temperatures, and hold for 5 min. Ice was manually seeded in the straws during the hold (if not already self-seeded) and the straws were further cooled at various rates in the freezers to -35 ^{o}C before being plunged into liquid nitrogen. For thawing, straws were placed in a water bath at 28 ^{o}C until the ice had melted (~ 5 sec), individually diluted 1:1 with seawater, left for 30 min, further diluted 1:9 with seawater and left for 30 min before being fertilized.

The low molecular weight CPAs dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and methanol (MeOH), alone or in combination with the high molecular weight CPAs trehalose or polyvinylpyrrolidone-40 (PVP), were evaluated at a range of concentrations (Tervit et al. 2005). No oocytes were fertilized when MeOH was used as the

CPA. Highest fertilization rates were achieved when final concentrations of either 10% EG or 15% DMSO were used in the presence of 0.85% PVP in Milli-Q water (Figure 1). Further experiments with non-permeating CPAs showed no beneficial effects of their addition and Milli-Q water was found to be superior to seawater as a base medium for the CPAs.

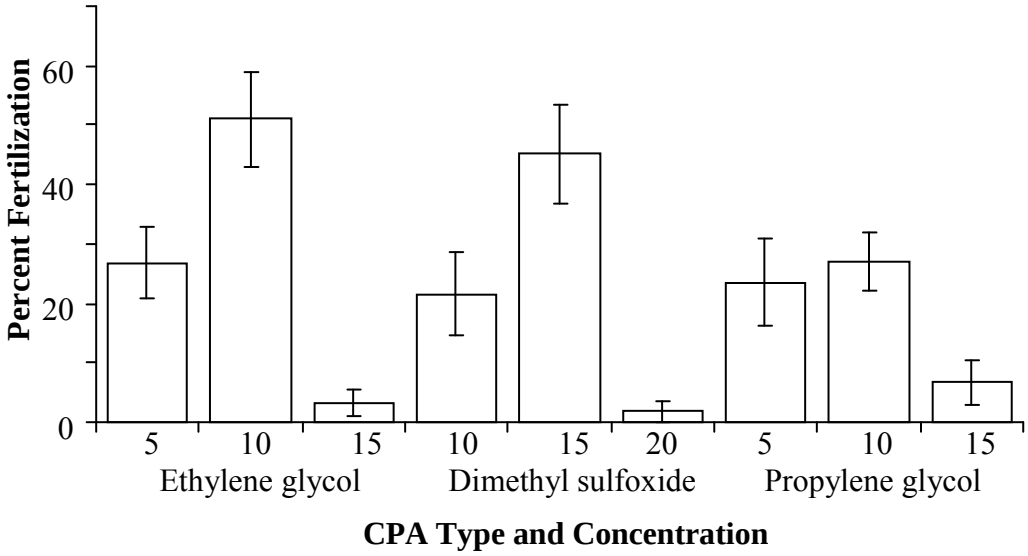

Figure 1. Effect of cryoprotectant (CPA) type and concentration (percent, v/v) on percent fertilization of thawed oocytes (mean ± SEM; n = 3 pools) (modified from Tervit et al. 2005).

Investigation of the effect of various holding temperatures showed that holding at -10 ^{o}C to -12 ^{o}C resulted in higher post-thaw fertilization than did higher holding temperatures or no hold (Tervit et al. 2005) (Figure 2). The cooling rate between the hold temperature and plunge temperature was also found to be important (Tervit et al. 2005). Post-thaw fertilization was highest (49 ± 8%, mean ± SEM, n = 3 pools) when oocytes were cooled at 0.3 ^{o}C/min, the slowest cooling rate tested. At faster cooling rates, fertilization decreased significantly and was almost zero when cooling rates of 3 ^{o}C/min or faster were used (28 ± 7% at 1 ^{o}C/min; 0 ± 1% at 3 ^{o}C/min, and 0 ± 0% at 6 ^{o}C/min). The use of 10% EG (with or without 0.85% PVP) yielded oocytes that were capable of developing to larval and spat stages at rates similar to oocytes that were not cryopreserved (Tervit et al. 2005). However, variation in post-thaw fertilization between females (Figure 3) and between straws remains an issue and further refinement of the method is needed before it can be reliably applied by selective breeding programs.

Therefore the cryopreservation method was examined to determine the critical steps affecting post-thaw fertilization and D-stage development. Because oocyte permeability characteristics suggested that there would be little difference in the volume changes experienced when single-step addition rather than ten-step addition was used (Salinas-Flores et al. 2008a), EG was loaded in a single step to give a final concentration of 10%. The oocytes appeared to tolerate CPA addition well but show marked developmental losses around -10 ^{o}C and between this temperature and -35 ^{o}C (Figure 4). A feature of the results was the poor development of

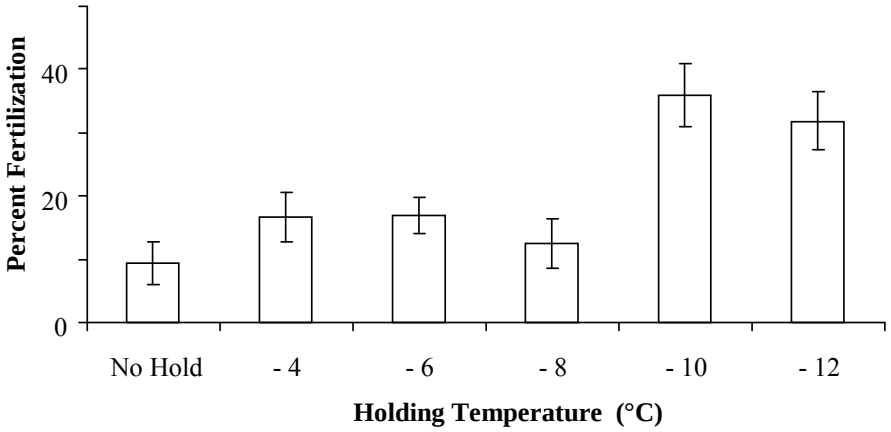

Figure 2. Effect of the isothermic holding temperature on percent fertilization of post-thaw oocytes (n = 3 pools) (modified from Tervit et al. 2005).

fertilized oocytes to D-stage larvae. The loss pattern suggests that "slow cooling" injury was occurring between -10 and -35 ^{o}C. However, studies have shown that intracellular ice is present following cooling to liquid nitrogen temperature using this regime (Salinas-Flores et al. 2008b). In that study, ice was not observed by use of a cryomicroscope at temperatures between -10 and -35 ^{o}C following cooling at 0.3 ^{o}C/min (perhaps because of the high lipid and yolk content of the oocytes or because of the small size of the ice crystals that formed); however transmission electron microscopy of samples that had been taken to liquid nitrogen temperature showed ice throughout the cells. Therefore, it is likely that slow and fast cooling injuries occurred in these cells using this cooling protocol.

Attempts were made to improve the protocol by using a slower cooling rate (0.1 ^{o}C/min), lower plunge temperature (-60 ^{o}C) and incorporation of a 30-min hold at the plunge temperature (Salinas-Flores et al. 2008b). However, none of these changes resulted in an improvement in post-thaw fertilization. If anything, post-thaw fertilization was reduced, particularly when plunging at -60 ^{o}C rather than at -35 ^{o}C (Table 1).

Table 1. Post-thaw fertilization of oyster oocytes (n = 5) (mean ± SEM). Values sharing superscripts were not significantly different ($P < 0.05$). Samples were held at 0 ^{o}C for 5 min, cooled to -10 ^{o}C at 1 ^{o}C/min, held for 5 min (and seeded if necessary) before cooling using the various parameters. (Modified from Salinas-Flores et al. 2008b).

Cooling rate (^{o}C/min)	Plunge temperature	Holding time at plunge temperature (min)	Percent fertilization
0.3	-35 ^{o}C	0	42 ± 6 ^{a}
0.3	-35 ^{o}C	30	41 ± 6 ^{a}
0.3	-60 ^{o}C	0	17 ± 4 $^{b, c}$
0.3	-60 ^{o}C	30	12 ± 6 ^{c}
0.1	-35 ^{o}C	0	31 ± 3 $^{a, b}$
0.1	-35 ^{o}C	30	43 ± 2 ^{a}
0.1	-60 ^{o}C	0	9 ± 1 ^{c}
0.1	-60 ^{o}C	30	6 ± 1 ^{c}

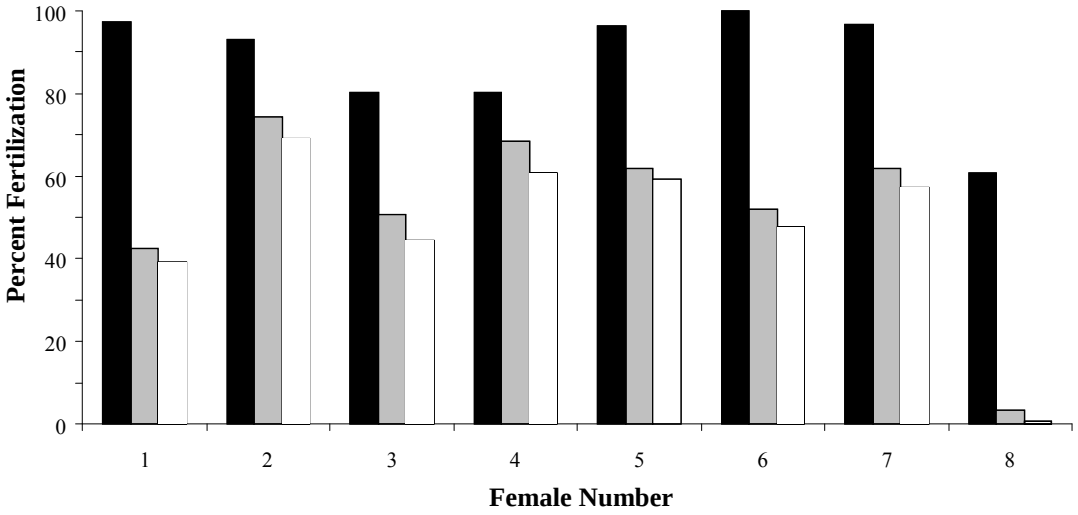

Figure 3. Percent fertilization from 8 individual females of non-frozen oocytes (black bars) and oocytes cryopreserved in 10% ethylene glycol without (grey bars) or with (white bars) polyvinylpyrrolidone-40 (from Tervit et al. 2005).

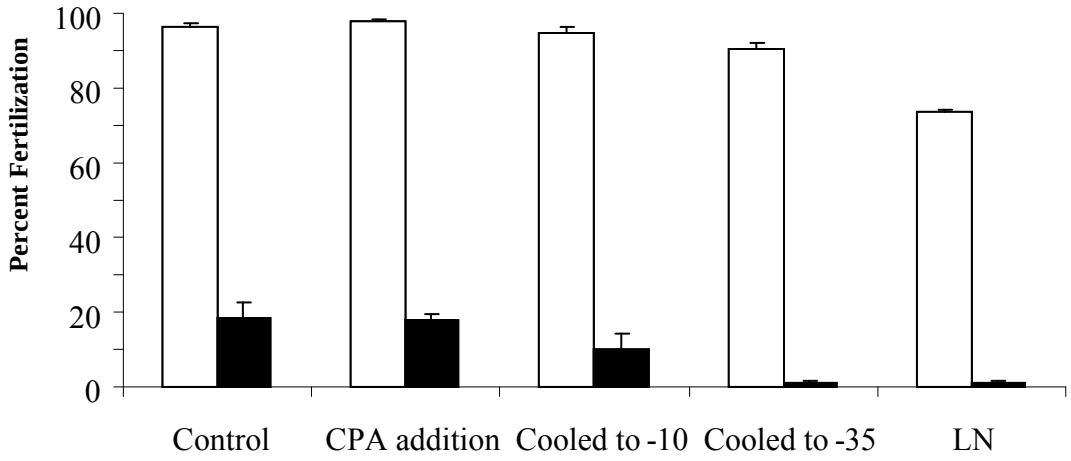

Figure 4. Effect of each cryopreservation step on percent fertilization (white bars) and yield of D-stage larvae (black bars) of oocytes frozen in 10% ethylene glycol (mean ± SEM; n = 3 pools).

Experiments to manipulate the composition of the oocytes to improve freezing response have also been undertaken (Salinas-Flores 2007, Salinas-Flores et al. 2008c). In these studies, attempts were made to either directly manipulate oocyte composition through incubation in media enriched with cholesterol or fatty acid (eicosapentaenoic acid) (EPA) or docosahexaenoic acid (DHA), or indirectly manipulate composition by feeding broodstock different diets during conditioning. Oocytes incubated in cholesterol-rich solutions showed a positive uptake of fluorescent labeled cholesterol which was dose dependent. However, the total cholesterol content of the oocytes was unchanged and there was no improvement in post-thaw fertilization rates (Salinas-Flores 2007, Salinas-Flores et al. 2008c). Oocytes incubated in non-conjugated or

conjugated EPA or DHA failed to show any change in the proportion of these fatty acids in their profiles and no improvement in post-thaw fertilization was observed (Salinas-Flores 2007). Conditioning of broodstock on different diets altered the total lipid content of the oocytes as well as the amount and type of polyunsaturated fatty acids present. Overall, animals that were fed micro-algae during the gametogenesis phase of conditioning produced oocytes that were more amenable to freezing than animals that were starved during this phase with post-thaw fertilization rates 5-10% higher (Salinas-Flores 2007).

The effect of oocyte nuclear stage has been examined for influences on post-thaw viability. The addition of serotonin to seawater has been shown to induce maturation of Pacific oyster oocytes to metaphase I (Leclerc et al. 2000) while calcium-free seawater can help maintain the oocytes in prophase, although acidified seawater works better (our unpublished observation). Oocytes from three individuals were stripped into either seawater or calcium-free artificial seawater. Some of the oocytes stripped into seawater were transferred to seawater containing 8×10^{-6} M serotonin. The oocytes were held in each solution for 2 hr before freezing. Nuclear maturation showed that holding for 2 hr in serotonin decreased the percentage of oocytes in prophase (from: 94% to 74% in individual 1; 76% to 4%, individual 2; 93% to 50%, individual 3). In calcium-free seawater, the percentage in prophase was not different from the percentage in natural seawater (98%, 85% and 94% for individuals 1, 2, and 3) and there was no effect of serotonin or calcium presence on post-thaw fertilization (8 ± 7% for seawater (n = 3 individuals); 23 ±14% for serotonin, and 10 ± 7% for calcium-free seawater). A second experiment utilized spawned or stripped oocytes from 27 females. The percentage of oocytes in prophase, metaphase I (or intermediate between these stages) at the time of freezing and the post-thaw fertilization success of the oocytes was determined. Regression analyses showed no relationship between the percentage of oocytes in either prophase ($R^2 = 0.0406$) or metaphase ($R^2 = 0.00006$) and the post-thaw fertilization of oocytes.

Conclusions

The ability to cryopreserve Pacific oyster oocytes will make a significant contribution to selective breeding programs and commercial shellfish hatcheries. Successful cryopreservation has been achieved with tens of thousands of spat produced from thawed oocytes. However, extreme variation in the viability of the oocytes post-thaw presently limits the method from being reliably applied. Further fundamental and applied research is being carried out to improve the method and enable its routine implementation and use in breeding programs and hatcheries.

Acknowledgments

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New Chapter

Conserving Coral Reefs

Mary Hagedorn

Introduction

Coral reefs are living, dynamic ecosystems. They are the rainforest of our oceans and have been on our planet for more than 200 million years. Although all of the reefs in the world only occupy an area the size of the country of Bolivia, they are small eco-dynamos. They provide invaluable services to us, such as acting as nursery grounds for marine fish and invertebrates, providing natural storm barriers for coastlines, purifying CO_2 from the atmosphere, and could provide potential sources for undiscovered pharmaceuticals.

Unfortunately, coral reefs are experiencing unprecedented levels of degradation due to the impact of humans (Bellwood et al. 2004, Kleypas et al. 2006). Globally, increased levels of greenhouse gases are warming our oceans, making them more acidic and causing the coral to stress, bleach, and be more susceptible to newly emergent diseases (Hoegh-Guldberg 1999, Goreau et al. 2000; Hughes et al. 2003). Locally, reefs are impacted by pollution and sedimentation from poor land-use practices, nutrients from farms and waste treatment plants, and destructive practices, such as impacts to the reef from dynamite fishing, trawls, and boating. Unless we take action now, coral reefs and many of their associated animals may cease to exist within the next 40 yr, causing the first global extinction of a worldwide ecosystem within our history (Veron et al. 2009).

Each year, coral reefs contribute as much as $US 30 billion to the global economy (Cesar et al. 2003), but no one knows how the loss of coral reefs might affect our global ecosystems and economies because we do not understand enough about the ecological and economic multipliers related to reefs. For example, at some point in their lifetime, about 25% of all marine creatures live on a reef (Cesar et al. 2003). When you think of the billions of individuals in a single species, then multiply it by the thousands of species that live directly on a reef at any one time, it is clear that reefs support an extraordinary amount of biomass. How many other species depend upon this 25% for their survival is not known, but what is known is that, if reefs were to disappear, many of the fish that we depend upon as essential food sources today will become luxury items. According to Cassandra de Young of the United Nations Food and Agricultural Organization, with over 1 billion people on the planet already hungry, the disappearance of seafood may cause great instability in food supplies around the world.

Caribbean reefs are suffering the most, and their fate may predict the future of the corals throughout the world. For example, *Acropora palmata* (elkhorn coral) and *Acropora cervicornis* (staghorn coral) were once the foundation species for Caribbean reefs, but are no longer fully functional ecosystems (Alvarez-Filip et al. 2009, Gardner et al. 2003, Buddemeier et al. 2003). This is due to the enormous population loss (80-99%) in these species from their historical levels (Aronson and Precht 2001, Bruckner 2002), and for the first time, corals (elkhorn and staghorn) are listed as threatened under the Endangered Species Act (Federal Register 2006, Federal Register 2008 a, b). Recovery of existing populations depends on growth rates and reproduction. If we focus just on reproduction there are some alarming trends in these critical species. First, coral can reproduce sexually which increases genetic diversity, and they can also reproduce

Hagedorn, M. 2011. Conserving Coral Reefs. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 624-630.

asexually which does not increase diversity. In the Caribbean, *A. palmata* and *A. cervicornis* seem to reproduce more commonly through asexual breakage and reattachment of branches than through the production of eggs and sperm (Highsmith 1982, Williams et al. 2009). Therefore, spawning may be uncommon events or under-reported for many isolated, stressed, damaged, or small populations, especially the remaining acroporids in the Caribbean. During spawning, fertilization success is directly dependent upon the genetic diversity of neighboring colonies, and the genetic diversity in some areas has already declined, such as in the Florida Keys (Baums et al. 2006). These remnant populations are facing severe problems because they will be very susceptible to extinction over the next 5-20 yr (Zubillaga et al. 2008, Macintyre and Toscano 2007). Additionally, recruits in these acroporid populations suffer high mortality rates, because of a lack of suitable habitat at the early post-settlement stages, causing further population declines for both species. In brief, numbers of acroporid colonies are declining, and even populations that still exist are having difficulties with reproduction, recruitment and growth (Grober-Dunsmore et al. 2006, Nzali et al. 1998). While the predicted global failure of our reefs is based on the coastal waters becoming more acidic and actually dissolving when the CO_2 in the atmosphere rises above 450 ppm (Veron et al. 2009), if the failing reproductive and recruitment patterns observed in the Caribbean are harbingers for reefs in other oceans, then the prediction of 40 yr for the global loss of our reefs may be an overestimate.

Live and Frozen Repositories

There is a widespread crisis facing coral reefs and an urgent need for effective conservation action. The Species Survival Commission of the International Union for Conservation of Nature and Natural Resources (IUCN) has identified corals as one of the planet's three major species extinction crises. Unfortunately, saving reef habitat alone will not stop this decline, because many of the most serious threats are global rather than local. Genetic banks comprised of live or frozen biomaterials hold strong promise for basic and applied research and conservation of species and genetic variation (Ballou 1992, Wildt et al. 1997, Holt and Pickard 1999). Because frozen banked cells are viable, the frozen material can be thawed 1, 50, or, in theory, even 1,000 yr from now to restore a species or population. Done properly over time, samples of frozen material can be reared and placed back into ecosystems to infuse new genes and vigor into natural populations, thereby helping to enhance the health and viability of wild stocks (Dobson and Lyles 2000, Wolfe et al. 2001, Wildt et al. 2001). Most examples of using frozen germplasm for enhancing genetic management of small populations are for mammals (see review by Pukazhenthi and Wildt 2004). These groups used frozen samples to diversify wild and captive populations. These resource banks can: (1) easily and inexpensively move genetic material among living populations; (2) extend generational intervals for genetically valuable individuals; (3) reduce space constraints for *ex situ* populations; and (4) vastly improve the availability of biomaterials for scholarly research, including in disease forensics (Wildt et al. 1997, Holt and Pickard 1999). A repository for the world's most endangered coral species has now been developed. The Smithsonian, in collaboration with many partner organizations (e.g., Hawaii Institute of Marine Biology, Louisiana State University Agricultural Center, Sexual Coral Reproduction (SECORE), Henry Doorly Zoo, USDA National Animal Germplasm Program and the Center for Genetic Resources in the Netherlands), has created the first frozen repository for coral in the world (Hagedorn et al. unpublished). This group has achieved several important milestones over the past 5 yr in coral physiology and cryopreservation (Hagedorn et al.

2006, 2010, Yancey et al. 2010), including being the only group to cryopreserve coral sperm (Hagedorn et al. 2006), cryopreserve coral embryonic cells (Hagedorn et al. unpublished), produce viable coral larvae from thawed cryopreserved sperm, as well as examining the cryosensitivity and cryopreservation of the symbiotic algae living within the coral (Hagedorn et al. 2010). In addition, this research consortium has been actively involved in conservation of endangered species, designing many of the larval rearing chambers needed for growing endangered coral larvae in captivity, and applying these techniques under field conditions in the Caribbean (Hagedorn et al. 2009). These distributed coral banks contain viable sperm and embryonic cells from *A. palmata* and 2 Hawaiian species, with additional species to be added in the future (see Fig. 1). The frozen sperm may be used to increase genetic diversity within an ecosystem through the use of thawed samples to 'seed' and restore shrinking populations. A primary focus might be the declining *A. palmata* populations in the Florida Keys, where many stands are genetically identical (Baums et al. 2005), yielding an extremely inbred population.

Figure 1. The mushroom coral *Fungia scutaria* is one of the species in the frozen repository for Hawaiian coral.

Public zoos and aquariums can play a crucial role in saving reefs by educating the public and creating live banks of coral, containing either sexual recruits or small coral fragments. A good example of these emerging cooperative efforts among public zoos and aquariums is SECORE. The Rotterdam Zoo initiated SECORE in 2001 to study coral sexual reproduction; develop *ex situ* breeding techniques; disseminate these techniques among aquarium and research communities through workshops and publications; develop a cooperative international network of public aquariums and research institutions, and establish breeding programs to help sustain *ex situ* and field populations. In 2006 and 2007, SECORE members, representing national and international institutions, held highly successful research workshops in Puerto Rico to rear Elkhorn coral from the annual mass spawning events at Rincón and Bajo Gallardo sites. Close to 1 million larvae were collected and fertilized; hundreds of thousands were raised in a field laboratory; and over 400,000 brought into captivity, resulting in approximately 2,300 juvenile larval recruits now living in public aquariums around the world (Petersen et al. 2006, 2007). These were the first juveniles of this species ever reared in captivity, and represent a major step towards the restoration of this species in the wild. Live coral banks can be created now, with little or no increase in infrastructure costs. The small fragments in the bank could be kept alive

for hundreds of years and, because there would be no reproduction, there would be no genetic drift in the populations stored around the world. Moreover, these insurance populations in the live banks could potentially be used to 'reseed the oceans', if the oceans were suitably rehabilitated.

Call to Action

It is not an ideal scenario to have to consider freezing reef fragments, or putting small coral pieces into captivity, but the possibility of losing such precious ecosystems from our planet is unthinkable. Therefore, we need to plan for the future and act now, before it is too late. Support for studies to understand the physiology and molecular biology to cryopreserve coral cells and fragments that are robust (post-thaw) and genetically diverse is imperative. These programs must promote the training of graduate students and postdoctoral fellows in coral physiology, conservation and cryobiology. Additionally, in coordination with geneticists and conservation biologists, public zoos and aquariums around the world must create a coordinated global plan for long-term *ex situ* conservation and maintenance of targeted species, using the best practices and internationally agreed upon husbandry techniques. Many zoos and aquariums are already experts at maintaining coral, and these institutions could have a rapid and profound impact on sustaining reefs by maintaining select species in captivity in a coordinated program around the world.

We need the broad practical knowledge that already exists in zoos and aquariums around the world to help save our reefs. We urge these groups to form teams, hold workshops to create partnerships and working groups and get connect by joining SECORE *(www.secore.org)*. It is critical that a worldwide plan be put into place to standardize cryopreservation methods throughout the world. Several areas, such as Europe, the USA, Australia and Indonesia could serve as interconnected banks that not only hold material, but train professionals in countries that have coral and would like to form national banks. Paraprofessional or professional aquarists could choose to support a series of live banks of small fragments from one or two species in live culture. However, the methods for care, tagging, handling, genetic identification and survivorship (fragments might reside in captivity for decades) of these banks would have to be established. With relatively small investments, these live banks could be formed today, and would have an enormous impact on ensuring the continued diversity of our reefs. As humans, we tend not to act until disaster is upon our doorstep, threatening our very existence. However, if we wait as the escalating anthropogenic threats and natural processes ravage coral populations, leaving little remnant genetic diversity in their wake, we cannot predict precisely what effect that will have on our global ecosystem. But, to put simply, it will not be good. We must not let one of the oldest and most beautiful ecosystems on our planet disappear, because we could not find the will to act in time.

Acknowledgments

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Cryopreservation of Juveniles of a Marine Polychaete

Peter J. W. Olive and Wen B. Wang

The procedures described here are subject to patent protection as applied to the aquaculture of marine annelids for use as bait. Permission to license the use of the methods described for this purpose should be addressed to Seabait Limited, Woodhorn Village, Ashington, Northumberland NE63 9NW UK or to the first author.

Introduction

A protocol allowing the mass preservation of fully differentiated nechtochaete larvae of the polychaete *Nereis virens* has been developed (Wang and Olive 1996, Olive and Wang 1997). This represents a significant step in the application of cryobiological techniques to aquatic organisms. At the stage at which the juvenile Nereidae can be preserved, they have completed the embryonic phases of development and are independent organisms functional in the adult habitat. Cryopreservation of whole organisms is intrinsically more challenging than single tissues and organs due to their structural complexity (Mazur 1984, Pegg 1994, Mazur 1996).

Intensive aquaculture systems for polychaetes have developed to meet demand for production of sea-angling live bait (Creaser and Clifford 1982, Olive et al. 1992, Brown 1993, Olive 1993), for use as food organisms in aquaculture (Lytle et al. 1990, Olive et al. 1992, Olive 1984), as materials for scientific investigation and whole organism toxicity testing (Reish 1980, Rubinstein et al. 1983, Olla et al. 1984, Jensen and Baatrup 1988, Jenner and Bowmer 1992, Pruell et al. 1993, Miron et al. 1994) and most recently sublethal cytological toxicity testing (Hutchinson et al. 1995, Jha et al. 1995, Jha et al. 1996, Hutchinson et al. 1998). A large-scale culture system was developed by Seabait Ltd., United Kingdom (UK) following an initial transfer of technology from the University of Newcastle (UK) and this has provided the impetus for the exploration of application of cryopreservation techniques to these organisms. The reproductive biology of most larger polychaetes involves a highly synchronized breeding cycle in which gravid animals are present under natural conditions only within a short breeding season (Bentley and Pacey 1992, Olive 1984, 1997). Such a situation provides only a narrow window of opportunity for the production of larvae for supply to aquaculture systems, for use in toxicity tests and for other scientific applications. This represents a significant constraint on commercial opportunities.

Cryopreservation of the gametes and larvae of polychaetes offer a means of overcoming this constraint. The principle findings are presented here, but procedures relating to the cryopreservation of polychaete larvae in polychaete aquaculture applications are subject to patent. The investigation has included studies of male and female gametes as well as developmental stages, but the greatest success has been achieved with juvenile worms derived from induced breeding of captive animals. Because a female *N. virens* is able to provide up to 1 million simultaneously mature eggs, this represents a valuable source of preserved animals at an advanced stage of development with the potential for use in many applications.

Olive, P. J. W. and W. B. Wang. 2011. Cryopreservation of Juveniles of a Marine Polychaete. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 631-644.

Embryonic and Larval Development in *Nereis virens*

The success of techniques for the cryopreservation of *N. virens* is highly dependent on the stage of development and a brief description of the development of *Nereis* will be useful. The fully mature oocyte has a diameter of 200 μm, is richly provisioned with lipid and protein yolk and has a concentric structure with a prominent cortical band of mucopolysacharide material from which is derived the massively thick (200 μm) layer of jelly material emitted on fertilization. The sperm of Nereidae are of a modified ect-aquasperm type (Rouse 1995) with a prominent acrosome pushing into a deep indentation into the head and nucleus of the sperm. The sperm have been successfully cryopreserved (Bury and Olive 1993) though with some membrane damage. The sexes are separate and males and females each become fully gravid prior to a short breeding season normally in the northern hemisphere spring (April to May). Like all Nereidae, these animals are strictly semelparous and do not survive breeding; as a consequence a large amount of germinal tissue is produced and a 5-g gravid female can contain in excess of 1 million eggs representing some 70% of the body mass.

Fertilization occurs while the oocytes remain in prophase I of meiosis. Cleavage follows a spiral pattern with a large D-cell lineage. The vegetal pole cells are large and receive most of the lipid yolk present in the egg. As a consequence, the blastula is solid and does not have an open blastocoele. Gastrulation reflects the high yolk content of the egg and involves an epibolic spreading of the animal pole ectodermal cells over the lipid-yolk filled cells of the vegetal pole leading to the invagination of a prominent cell from which the mesodermal elements of future adult segments are derived. The embryo becomes a trochophore and has a brief free-living phase. The trochophore shows regional differentiation into pre-prototrochal prostomium, prototroch, three larval chaetigerous segments derived from post-prototrochal cells of the trochophore, a pre-pygidial segment proliferation zone and the post segmental pygidium (Bass and Brafield 1972). The first three larval chaetigerous segments thus have a different embryonic origin from the adult segments and of these the first subsequently becomes achaetigerous (Figure 1). The establishment of the pre-pygidial proliferation zone from which adult segments are progressively delaminated represents an important step in development. It is from the cells of this region that all the future segments are derived. After the formation of the prepygidial segment proliferation zone, the series of about 120 adult segments is progressively produced (Olive 1974). The four segment *N. virens* has in effect established the adult body plan, it exhibits precocious functionality in the three larval segments, has largely reabsorbed stored yolk nutrients and is ready to feed. Soon after the formation of the three larval chaetigers, the larvae enter an exploratory phase in which there is alternation of swimming and crawling behavior; the larvae enter the adult environment about 10 d after fertilization at 12 ^{o}C. The yolk supplies derived from the macromeres of the embryo are allocated to the posterior gut cells and are progressively converted and used during the pelagic and early benthonic stages of development.

Larval Development and Sensitivity to Cryopreservation Protocols in *N. Virens*

The successful preservation of juvenile *N. virens* depends on two critical aspects: the stage of development of the larvae and, the precise protocols adopted to prevent internal ice formation during cooling. A systematic survey of the application of a potentially successful protocol during the stages of development of *N. virens* has been carried out taking care to sustain

the identical cooling and preservation protocols (for full details see Olive and Wang 1997). Sub-samples from batches of larvae derived from a single fertilization were investigated and the pattern of survival changed in a similar way from egg to the post-nechtochaete larval stage.

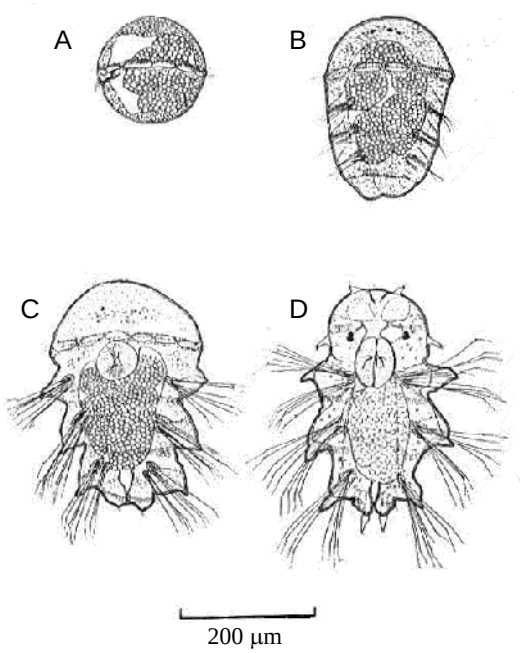

Figure 1. Stages in the development of *Nereis virens* from Bass and Brafield (1972). A: Early swimming trochophore. B & C : Three setiger trochophore exhibiting the early formation of three larval segments. D: Nechtochaete larvae at a stage of development approaching that when cryopreservation protocols are most successful.

Eggs and pre-trochophore larval stages could not be preserved using the protocols adopted, but once the first larval segments appeared there was an increase, then a decrease in the rate of survival. Peak survival occurred around 10 d of development at 12 ^{o}C and, in the best batches of larvae, the number of larvae surviving cryopreservation at this age would approach 100%.

The critically important developmental events occurring at this time are thought to affect the developing tissues of the alimentary system (Wang and Olive 1999). There is at this time a progressive decrease in the size of the lipid spheres in the cells of the hind-gut and regional development of the alimentary system occurs. The muscular pharynx develops in front of the yolk filled mid-gut cells and becomes physically connected to the hind-gut after 10 d of development. The narrow hind-gut establishes the exit via the pygidium and the lumen of the gut is present by about 10 d and the appearance of a clearly demarcated central line in the mid-gut is a good indicator that the optimum stage of development for preservation has been reached.

At this stage the prepygidial growth zone has developed and the fourth segment, the first of the adult segments, is present. The first three segments now have prominent parapodial structures including intrinsic musculature for the operation and movement of the chaetae and aciculum. The worm by now is in effect a miniature adult although it retains a number of larval

features including pigmented patches on the prostomium prototrochal cilia and the ability to move by ciliary gliding. The normal mode of movement is however by muscular movements and paddling. The gut is capable of independent peristaltic movements and the pharynx is eversible.

After 12 d of development, a progressive loss of viability was observed, and after some 20 d of development successful preservation proved difficult to achieve. During this time the animals had started to feed and would almost certainly have ice nucleation proteins in the developing gut lumen.

The Cryopreservation Protocol for *Nereis*

A systematic survey of cryopreservation protocols has been undertaken following an investigation of the kinetics of water movement through the boundary layers of larvae and eggs of *N. virens*. The most successful levels of preservation and recovery were achieved using a progressive cooling of larvae equilibrated with 10% DMSO in seawater. The rate of cooling is critical, a cooling rate of 2.5 °C per min from room temperature being optimal in 0.5-mL straws. Progressive cooling to a pre-quench temperature of -35 °C prior to plunging into LN_2 was the empirically derived optimum protocol**.**

Light microscopy during a simulation of this procedure on a Planar cryostage microscope revealed that ice formation occurred only in the external medium at a temperature of around -12 ^{o}C and that in most instances the tissues of the larvae did not freeze as the ice front passed over the observed larvae (Figure 2). Larval freezing could, if conditions were not appropriate, be observed as a “black flashing” event, but did not occur under ideal cooling conditions. When, as in Figure 2, the embryo itself does not freeze during cooling, it is surrounded by an ice and solute matrix and, as the temperature is reduced, the concentration of extracellular solutes in the unfrozen medium increases, drawing water from the biological subject which thus becomes progressively dehydrated.

The concentration (osmolality) of the external medium is given by the relation:

$$C = \Delta T / 1.86$$

Where C is by definition external osmolality and ΔT is number of degrees below 0 °C. The value 1.86 is the molal freezing-point depression constant for water (Mazur 1984). In partly frozen solutions, C is independent both of the nature of the solutes and of their total concentration prior to freezing. The equilibrium osmolality of the unfrozen fraction is a function of temperature only. Thus at -20 °C, external osmolality should be 20/1.86 = 10.75 Osmol/Kg.

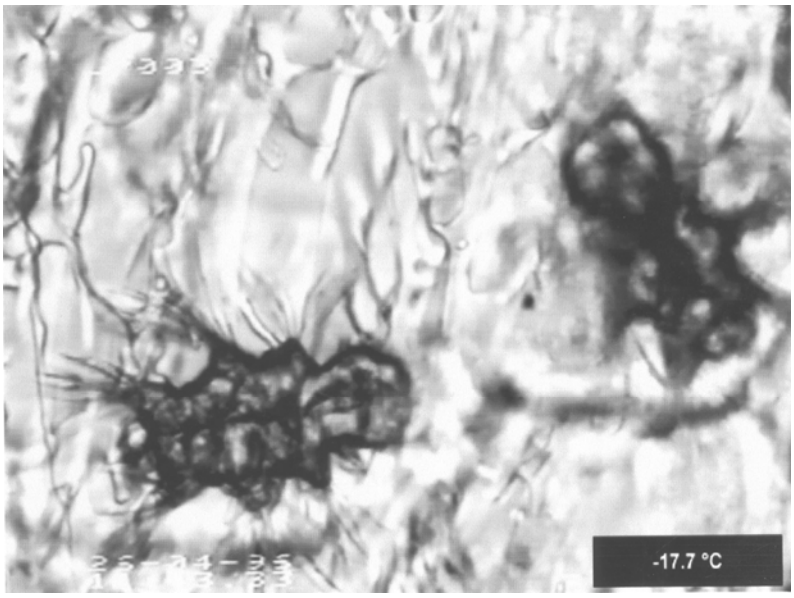

Figure 2. A light cryomicrograph image of unfrozen larvae in the presence of an extacellular ice matrix as observed with the Planar cryomicroscope stage taken from a real-time video recording using an Olympus BH2 microscope. The ice field typically forms at about -12 °C. The formation of ice within the larvae is made evident by sudden darkening of the tissues (black flashing).

Under equilibrium conditions, the water potential gradient across the outer boundary of the subject will cause a net efflux of water from the tissues and other osmotic compartments of the animal and loss of water occurs progressively as the temperature is reduced. Such loss of water can result in two terminal conditions which will allow quenching in LN_2 without ice formation and thus without loss of biological viability: 1) the free water in the biological system is reduced to <6.5% of the free water volume at room temperature, and 2) the concentration of cryopreservation agent present is sufficient to vitrify during the rapid cooling undergone by the biological subject during quenching. The kinetics of water loss from eggs and larvae of *N. virens* have been established at room temperature by equilibration with salt and sugar solutions of different osmolarities (Figure 3).

Under equilibrium cooling conditions, critical conditions should occur at around -25 °C; the observation that further cooling to -35 °C resulted in a significant improvement in the survival rate after recovery from LN_2 suggests that there is a degree of departure from equilibrium when cooling at 2.5 °C per min. This would retain water and perhaps allow ice nucleation and prevent vitrification of the DMSO solute mixture in the biological compartments of the subject when cooled only to -25 °C. A reduction in the cooling rate which would reduce the non-equilibrium effect does not, however, result in increased viability. This is probably due to the increased exposure to high concentrations of DMSO which, at room temperature, are lethal at the implied concentrations.

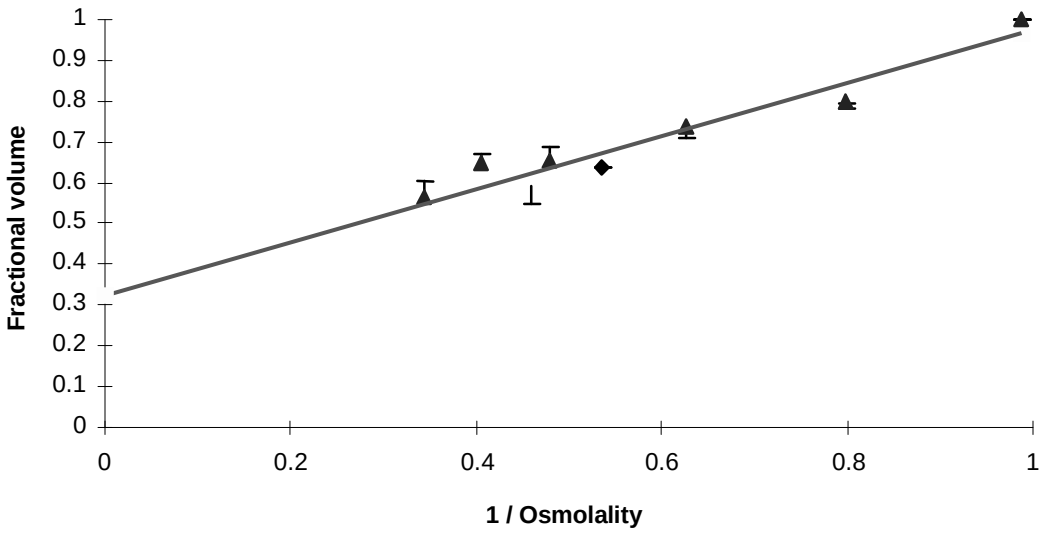

Figure 3. Osmometric behavior of three-segment larvae of *Nereis virens* in different hypertonic saline solutions. Bars show the standard deviations. The larvae closely follow the Boyle-van't Hoff relation V/Vo=0.332+0.653 C^{-1}. The coefficient of correlation for the best-fit equation was 0.95. The fractional volume is the volume of the larvae expressed as a fraction of its volume under isotonic conditions.

The optimum procedure for the preservation of the larvae of *N. virens* represents a compromise between opposing sets of conditions: 1) slow cooling to allow close adherance to equilibrium conditions and progressive water loss, and 2) increasing exposure time to high concentrations to toxic cryopreservation agents leading to increased mortality. More rapid cooling will result in greater departures from equilibrium and, at high rates of cooling, increased risk of ice formation within biological compartments as the ice front forms in the external medium around the tissues. Seeding ice formation in the external medium is, in this situation, unlikely to improve preservation of the tissues and may precipitate internal ice formation. The delicate balance between these factors and the need to find the optimum compromise is the likely reason for the variety of different protocols that have been developed for marine aquatic organisms (see below). The procedures will also be sensitive to the physical characteristics and dimensions of the system within which cooling occurs.

The Nature of Tissue Damage During Cryopreservation of Juvenile *Nereis*: A Model System with Multiple Osmotic Compartments

The cryopreserved larvae of *N. virens* are, we believe, the most developmentally advanced and complex whole organisms to be preserved to date. The objective of preserving fully differentiated organisms remains an important one and *Nereis* juveniles provide a useful model system for investigation of the causes of loss of viability in differentiated organisms during preservation.

A particular feature of this system is the relatively high salt content of the seawater medium and the presence in the larvae, when preservation reaches an optimum, of multiple extracellular osmotic compartments in addition to the osmotic compartments of the various of many individual cell types.

Use of a differential scanning calorimeter (DSC) has established that when individual larvae are cooled in parafin, in the absence of external water, ice nucleation within the larval tissues does not occur until the larvae are cooled to well below the normal ice nucleation temperature (Table 1).

Table 1. Differential Scanning Calorimeter measurements on individual 3-segment *Nereis virens* larvae in liquid paraffin.

Individual larvae Number	Freeze onset		Freeze enthalpy (mJ)	Freeze duration (sec)
	°K	°C		
1	241.8	-31.1	0.17	14.3
2	240.3	-32.8	0.19	14.3
3	239.4	-33.7	0.15	9.5
4	238.5	-34.6	0.12	9.5
5	234.7	-38.4	0.17	9.5
6	241.2	-31.9	0.15	9.6
7	240.6	-32.5	0.20	9.6
8	239.2	-33.9	0.16	9.5
9	238.0	-35.1	0.20	9.6
Mean ± SD	239.3 ± 2.1	-33.8	0.17 ± 0.03	10.6 ± 2.1

In the presence of external water, ice nucleation within larvae occurs at a higher temperature, at about the same temperature as the external medium, suggesting that cross membrane seeding of ice formation is potentially an important cause of cellular damage with consequences for the subsequent viability of the recovered animals.

A transmission electron microscope study of the cellular damage occurring during the low temperature dehydration and quenching of *N. virens* larvae and juveniles has been used to identify susceptible tissues within the complex system (Wang and Olive 1999). Tissue damage was primarily detected in the surface structures of the epithelium and in the cells of the developing alimentary system. The ciliated ectoderm of the larvae was sometimes visibly damaged during the procedures, although not all cilia were affected. Cryomicroscope observations of individual larvae cooled to -35 ^{o}C and subsequently returned to room temperature revealed immediate recovery of functional beating by the cilia. Loss of pigment from surface epithelial cells was also a frequent observation, but this was also induced by exposure to solutions of high osmolality without cooling, and was not found to be life threatening.

More complex internal epithelial structures such as the photoreceptors were not found to be damaged during cryopreservation. A high level of structural identity was retained in these structures (Figure 4), showing that membrane disruption can be prevented and internal cellular architecture can be retained. Similarly, the structure of muscle cells remained normal without obvious damage. Larvae were able to move within sec of return to room temperature from LN_2.

Damage to the cilia may be of greater importance in younger specimens as their ability to move would be reduced and the damaged cilia may be centers for microbial invasion, which could result in progressive mortality in recovered animals.

Our observations using light and electron microscopy indicated that the cells of the mid-gut were particularly susceptible to damage during cryopreservation and that this damage was the most likely cause of subsequent death. The mid-gut cells are derived from the four macromeres formed at the vegetal pole of the embryo at the third (1st transverse) cleavage and they have a high lipid content. The cells are filled with large lipid spheres and one of the obvious effects of preservation during the early

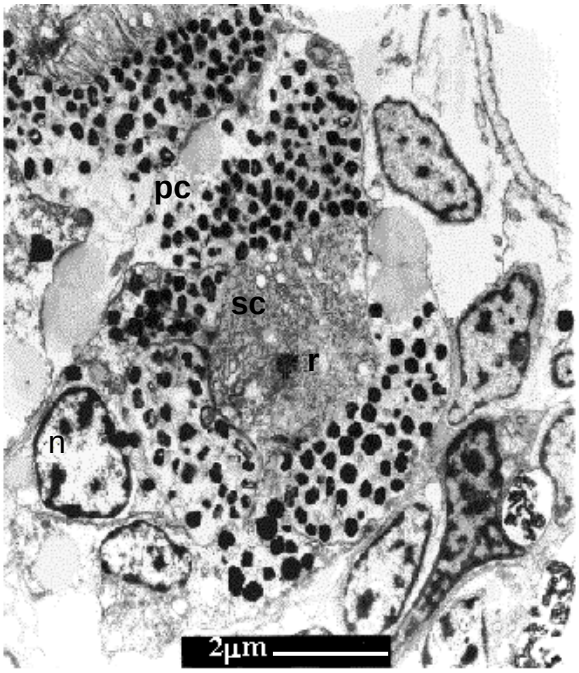

Figure 4. A transmission electron microscope image of the undamaged photoreceptor structures in the prostomium of a cryopreserved juvenile *Nereis virens* showing well preserved pigment cells (pc) and sensory cells (sc) with photoreceptoral processes and rootlet (r). The specimen was fixed in glutaldehyde and osmium tetroxide (Wang and Olive 1999) after recovery from LN_2 following preservation using the protocol described in detail in the international patent Publication No. WO98/06255.

phases of development is the formation of abnormally large lipid spheres which subsequently may leak into the newly formed coelomic spaces. If severe, such damage is always lethal (Olive and Wang 1997). As development proceeds, the lipid spheres become smaller and the number of gut cells increases at the same time cell volume decreases, resulting in larvae better able to withstand the cryopreservation procedure.

However, when the lumen of the gut has formed, this trend is reversed possibly because the gut lumen itself becomes an osmotic compartment likely to contain ice nucleation proteins.

Comparison of the *Nereis* Cryopreservation Protocol with those Applied to other Marine Invertebrates

A comparison of the procedures developed here with other studies of cryopreservation of marine invertebrate is now possible. A variety of cooling rates, cryoprotectants and cryoprotectant concentrations have been used with protocols for the preservation of marine invertebrate larvae. The diversity of approach reflects the rather empirical nature of many cryopreservation studies and there is a critical need to establish the theoretical basis of the approaches that have been adopted. Despite the great variation, there are some similarities among the protocols that have emerged. The stage of development of embryos and larvae is frequently observed to be critical. Two substances, DMSO and ethylene glycol (1 to 3 M), have most often been used as the cryoprotectant and good survival can be achieved using cooling rates between of 0.3 and 10 °C per min, although the optimum cooling rate is often between 2.0 to 5.0 °C per min. This suggests that in most cases the cooling rate adopted is faster than one that would allow strict equilibrium conditions. In such cases it may be necessary to cool to a prequenching temperature that is lower than would be the case for equilibrium cooling. The protocol adopted for *N. virens* follows this pattern, the optimum protocol reflecting a balance between the need for slow cooling to allow dehydration and more rapid cooling to avoid toxicity effects. Transfer to LN_2 is usually most successful at between -30 and -40 °C, which is rather lower than would be expected if the invertebrate larvae were equilibrating exactly with the external medium during cooling, and impendence of water loss may often be a problem. The effect of significant departures from equilibrium will be to increase the probability of internal ice formation. High survival rates have been reported with and without seeding procedures, but in many cases seeding was attempted at temperatures at which it was unlikely that the ice formed would have been stable. A comparison of the procedures developed here with other studies of cryopreservation of marine invertebrate is summarized in Table 2.

Conclusions

Can *Nereis virens* be considered as a model for the investigation of cryopreservation procedures in complex differentiated organisms? Probably not in the sense that the optimum protocol for *N. virens* larvae may prove to be an equally good protocol for all other species. However, a better understanding of the mechanisms behind the optimum protocols will help to make possible the systematic design of cryopreservation protocols and help in the search for the ideal protocols needed for the individual species. *Nereis virens* can produce up to a million eggs from a single fertilization, the larvae have a high degree of transparency and are easy to maintain before and after the cryopreservation procedure. All these features make *N. virens* an excellent animal for use as a model for study of cryopreservation of small aquatic organisms.

Table 2. Cryopreservation protocols applied to aquatic animals.

Scientific Name	Common name	Stage of development	Cryo – protectant*	Cooling rate (°C per min)	LN_2 plunge temperature (°C)	Seeding temperature (°C)	Survival rate (%)	Reference
Nereis virens	Ragworm	3-segment	1.4 M DMSO	2.5	-35	without	86	Olive and Wang 1997
Hemicentrotus pulcherrimus, Strongylocentrotus intermedius and *Loxechinus albus*	Sea urchin	Pluteus larvae	1.5 M EG or DMSO or 1.0 M DMSO	10.0	-150	without	76 - 100	Asahina and Takahashi 1978, Asahina and Takahashi 1979, Barros et al. 1996
Mytilus edulis	Blue mussel	Trochophore embryos	1.5 M DMSO	0.5	-30	-5	49	Toledo et al. 1989
Brachionus plicatilis	Rotifer	Symmetrical stage embryos	1.4M DMSO	0.3	-20	-5	63	Toledo and Kurokura 1990
Balanus Amphitrite	Barnacle	nauplii larvae	3 M EG	0.3	-40	-8	36	Anil et al. 1997
Crassostrea gigas and *Meretrix lusoria*	Pacific oyster and hard clam	late embryos and early larvae	2.0 M DMSO + 0.06 M Trehalose	2.0 - 5.0	-35	-12 or without	75 and 84	Chao et al. 1997

* DMSO, dimethyl sulfoxide; EG, ethylene glycol.

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Annotated Bibliography of Developments in the Last Decade

In our search the editorial board was unable to find further work in cryopreservation of juvenile polychaetes. However, there have been many studies and publications regarding cryopreservation of juveniles and embryos of other invertebrate phyla. Much success has also been realized in cryopreservation of numerous nematode species. These studies are valuable contributions as future preservation of invertebrate diversity is a key part of every ecosystem. A comprehensive review of cryopreservation of invertebrate embryos and juveniles could not be found, but is needed.

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VII. Some Basic Methods

New Chapter

Process Pathways for Cryopreservation Research, Application and Commercialization

Terrence R. Tiersch

Introduction

The bulk of all cryopreservation work in aquatic species over the past half century has addressed research-scale efforts at protocol development. This has yielded countless (often idiosyncratic) protocols that have been assembled in a number of outlets (e.g., Lahnsteiner 2000, Tiersch and Mazik 2000, Cabrita et al. 2009). To proceed into the next half century it will be useful to view the activities involved in cryopreservation as belonging to a series of process pathways that can be developed, refined and optimized, and also extended, broadened and envisioned in reverse. It is far less useful to collapse things down to empirical tinkering with the minimal cryobiological parameters (e.g., cryoprotectant and cooling rate) and to try to shoehorn application down this narrow research construct. By this I mean that if true commercial-scale cryopreservation is a goal, sample collection, refrigerated storage, and shipping methods will become just as important as cryoprotectant, and control of sperm-to-egg ratios and calculation of a fertilization unit will become just as important as cooling rate. The related activities that come before and after the actual freezing will be as important as the freezing in dictating overall cryopreservation success. Another important concept that emerges from developing a perspective based on multi-step pathways, is the recognition that cumulative damage (small, sequential, individually insignificant losses in gamete quality) can be just as debilitating to overall success as catastrophic damage (major quality losses that occur in only one or two failed steps).

Thus, cryopreservation becomes a *system of thinking and decision making* based on identification and integration of the activities necessary to propagate robust pathways. And, these process pathways can be configured in general or specific forms. This chapter will address three generalized, exemplary forms to serve as templates for future use and adaptation (Table 1, next page). The first pathway represents an ordered series of experiments and activities used to develop practical methods for species including those that are previously unstudied. It essentially is based on accepting samples in their arrival condition and trying to gain as much useful information as possible to identify problems and opportunities for that particular species. This is the basic approach followed in my laboratory for the past two decades in initial work with more than 100 species of fish and shellfish.

The second pathway builds directly upon the first by linking research results into standardized protocols that can be routinely applied. This pathway also includes additional research necessary to improve gamete quality before collection and after thawing, and thus inherently represents an optimization process focused on improvement of the entire process. *Although this includes optimization of individual steps, it should not do so at the expense of the overall results.* For example, use of a highly specialized container might provide an advantage in post-thaw motility, but if it compromised overall throughput (e.g., handling time), labeling quality, or storage efficiency this container would not be considered to offer overall improvement, and would not be incorporated in the application pathway (perhaps being reserved for research only).

Tiersch, T. R. 2011. Process Pathways for Cryopreservation Research, Application and Commercialization. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 646-671.

Table 1. Summary overview of three generalized inter-related pathways for the activities involved in initial development of cryopreservation protocols, small-scale application of the protocols, and commercial-scale application for eventual industry development for genetic resources of aquatic species. These pathways are addressed in more detail in other chapters (e.g., Hu and Tiersch, *Development of High-throughput Cryopreservation for Aquatic Species*). Research steps are indicated in bold.

A Research Pathway (Protocol development)	***An Application Pathway*** (Protocol utilization)	***A Commercialization Pathway*** (Programmatic development)
Gamete collection	**Improve male quality**	**Scaling up for high throughput**
Preliminary assessment	**Shipping**	**Commercial-scale facilities**
Initial motility estimation	Gamete collection	Gamete quality assessment
Dilution & choice of extender	Preliminary assessment	**Quality control & assurance programs**
Estimation of sperm concentration	Initial motility estimation	**Standardization & harmonization**
Motility duration	Estimation of sperm concentration	Establishment of markets
Osmotic & ionic activation curves	Dilution with extender	**Valuation & pricing schedules**
Refrigerated storage	Refrigerated storage & shipping	Providing product for customers
Acute toxicity	Cryoprotectant & equilibration	Providing services and training
Equilibration time	Equilibration motility	**Providing genetic improvement**
Equilibration motility	Labeling, packaging & cooling	**Providing equipment, supplies & reagents**
Packaging & cooling rate	**Storage, inventory & database**	Providing storage & shipping services
Thawing rate	Thawing & post-thaw amendments	Labeling, storage, inventory & databases
Post-thaw amendments	Post-thaw assessment	Biosecurity programs
Post-thaw assessment	Fertilization	Material transfer agreements
Fertilization assays	**Growth & survival**	Treaties & regulatory frameworks
Dilutions & concentrations	**Quality control points**	Access & benefit sharing agreements
Fertilization "unit" calculations	**Biosecurity protocols**	

The third pathway, unlike the first two, is mostly theoretical as we do not yet have large-scale commercialization in aquatic species cryopreservation, nor do we have industrial-scale markets for germplasm and genetic resources. Lastly, I will apologize here in advance for presenting the material below from a personal perspective based on our experience. These views are not universally shared (as is true for any specific research approach), but do represent a tested and reasonable starting point for others to build upon.

A Research Pathway (Protocol Development)

The basic methods and safety information presented in the previous original chapter (Wayman and Tiersch) did not require significant updating because not much has changed in these areas over the past decade. However, in light of the needs for application of technology, it will be useful as we go forward to focus on assembling these activities into standardized, practical platforms. The pathway described below introduces the steps that can be used to establish a basic protocol for a previously unstudied species, or to develop your own protocols after becoming familiar with the literature already available for that species or closely related taxa. However, beware: cryopreservation results are often difficult to transfer from one laboratory to another through published reports due to a burdensome lack of standardization in protocols and reporting (Tiersch et al. 2007, Yang et al. 2010). The collection and study of samples on-site or shipped from a remote location are addressed. We usually plan on addressing all of these activities and studies within a 2-d period with a two-person team, and can streamline the pathway if necessary to fit into a single, long day.

Gamete Collection

Usually sperm from ripe males can be obtained during the spawning season by either stripping or by crushing of dissected testis. Stripping of sperm involves collection directly from the male into a sterile tube. Care must be taken to avoid contamination of the sperm with dirt, feces, water, or urine. Dissection usually involves killing of the male and careful removal of the testis with scissors and forceps. Care must also be taken to avoid contamination with bacteria due to cutting of the intestine. Once removed, the testis is rinsed with extender (or the best guess as to something harmless such as isotonic saline for previously unstudied species) to remove blood, and extra tissues are dissected away. The cleaned testis is weighed and placed in a container with an appropriate amount of extender solution (typically a buffer) before it is crushed. The sperm are released and the solution is filtered to remove pieces of tissue.

Application of pressure under the pectoral fins is often sufficient to induce sperm flow in ripe males and avoids the problems associated with urine and fecal contamination caused by application of pressure along the abdomen in the traditional stripping motion (Tiersch et al. 1997). If working with an unstudied species for which there is no known extender, it may be necessary to aliquot each sample into two or three different extenders and to keep some undiluted to hedge your bets – this is easier at the start of a long spawning season, working with a plentiful species, rather than having a 3-d window to perform research on an endangered species in a remote location. As such, movement through this pathway needs to be and can be adjusted to suit the current situation of the research environment (i.e., there is no one way to do things, but the overall goal remains the same).

Decisions on whether to pool samples from individual males should be made with the overall goal of the project in mind. If the time and economic resources are available, it is most

informative to maintain the samples as separate individuals, especially for research. Alternatively, if the goal is for bulk production of fish, or if there are space limitations in a hatchery that would disallow maintenance of separate, multiple groups of fish it would make more sense to pool the samples. Pooling may also be necessary to provide sufficient volume for automated processing or to save time in processing. It should be noted, as described elsewhere in this volume, that pooling of sperm samples from several males is not a reliable method to produce genetic variation in offspring.

Preliminary Assessment of Samples

Samples should be evaluated when they are collected, before they are shipped, and again immediately upon receipt. With sperm, percent motility and general characteristics (e.g., color, presence of gelling) can be easily evaluated. The presence or absence of odors should be noted, and the temperature of the samples should be measured upon arrival. With experience, a quick assessment of sample quality and sperm concentration can be made by eye. It is important to discern if the samples could have been damaged or contaminated during shipment, for example by water from ice melt making its way into loosely sealed containers, or by inadvertent freezing due to use of -80 ^{o}C packaging (which is usually not necessary). It is often useful (probably essential) to include a sample of the ambient water the organism was collected from. This can be invaluable for the detective work associated with solving problems posed by new species or unanticipated results.

Initial Motility Estimation

Before proceeding with further work it is useful to "triage" samples immediately upon collection or receipt. In this way informed decisions can be made with respect to experimental design by identifying the number and quality of the available samples. A small aliquot of sample can be activated and assessed qualitatively for motility. It is important to ensure that sufficient dilution and mixing of sperm is used to elicit maximal activation for each sample. For estimation of percent motility, only sperm that are actively swimming in a forward motion should be included (some consider this a conservative estimate). Sperm that remain in place with only a vibratory movement should typically not be included (this can depend on the species). The procedure can be practiced to ensure that sperm movements are not due to swirling of the activating solution or random movement. Some microscopic organisms (e.g. bacteria) are motile and can be mistaken for sperm by inexperienced observers (Jenkins and Tiersch 1997).

It is important to identify if the samples are of poor quality early in the research process, and to try to elucidate where and when quality reductions occurred. This is especially important for shipped samples and the detective work will benefit from good communication with the sender. It is wasteful to pre-label 200 containers for an experiment planned for use of samples from ten males only to find that 8 of the samples were ruined by inadvertent freezing during shipping caused by poor packaging. It would be even more wasteful to unknowingly use the ruined samples for experiments that produce spurious results. It should be noted that sometimes samples arrive with poor motility, but can "rebound" somewhat over the next 24 hr. This is often the case for tubes that are completely filled without an airspace. Splitting the sample into two half-filled tubes can sometimes provide necessary aeration.

If working with an unstudied species, this is a good opportunity to make some preliminary observations related to choice of extender. These rough observations can be used to narrow down the appropriate test conditions for the next studies. For example, if aliquots of the

samples were placed into different experimental extenders upon collection, now is a good time to identify those that merit further study (and those that do not). Also it is important to be aware that in some cases, ambient environmental conditions can influence the motility characteristics of sperm. Fish collected at different sites or held under different conditions (such as salinity) can display variation in activation and duration of motility (Tiersch et al. unpublished). New species may require some detective work simply to identify conditions that produce sperm activation. A tip here is that if the sperm appear to be in good morphological condition, different activation solutions can be surveyed (including ambient water) and the slides topped with a coverslip. Activation can be delayed in some species such as a 30-min delay observed for coral sperm (Hagedorn et al. unpublished).

Dilution and Choice of Extender

The term "extender" refers to a solution of salts, sometimes including organic compounds such as sugars that help to maintain sperm viability prior to and during the freezing process (e.g., Hanks' balanced salt solution, HBSS). The nature of the effect of extenders is largely based on the control of osmotic pressure, pH, and ionic concentration as well as a supply of energy, and can extend the functional life and fertilizing capability of the sperm. Usually, the extender is a balanced salt buffer of specific pH and osmotic strength. Sometimes other components such as egg yolk and milk are added, but they usually offer little benefit and can interfere with viewing of the samples with a microscope.

Extenders have been developed for many species. A single extender, HBSS, has been used successfully in our laboratory with sperm of more than 100 species, but simple solutions such as 1% unbuffered salt (sodium chloride) have been used with good results at least for short-term (i.e., < 24 hr) storage. With appropriate testing, extenders can be prepared in large batches and be stored frozen until use. Use of extenders provides increased storage time and dilutes the sperm to a greater volume, making the sperm easier to work with. Extenders can be sterilized by passage through a filter or by autoclaving (if this does not affect the ingredients) and should be refrigerated. This is especially important for extenders that contain sugars. Antibiotics can be added to extenders to reduce the growth of bacteria that reduce sperm viability. Antibiotics can be toxic to sperm cells, and therefore should be optimized for each species. In addition, they can provide a false sense of security and lead to careless sample collection and handling. Dilution studies are placed ahead of sperm concentration estimation in the research pathway because undiluted sperm can rapidly lose quality (Jenkins et al. 2011). This must be established early for previously unstudied species.

Dilution after sperm collection is necessary to optimize the volume for efficient use, to counteract the effects of urine contamination, and for research of various factors. Dilution is often in the range of one part sperm to one to four parts of extender solution. However, excessive dilution of sperm samples (e.g., 1:50) has been found to reduce sperm motility in mammals, fish and oysters (e.g., Paniagua-Chavez et al. 1998). Samples collected by stripping can be contaminated by urine which can activate the sperm and reduce the storage lifetime. For example, urine of freshwater fishes is hypotonic to the body tissues and would activate sperm by reducing the osmotic pressure of the sample. Dilution of these samples in appropriate extender can counteract the activation by urine (usually by moving the osmotic pressure back to the isotonic range).

Samples collected by dissection and crushing of the testis may not benefit as much from dilution in extender due to the lack of urine contamination, and these samples may respond best

to a lower dilution ratio in extender, or no dilution (i.e., if a satisfactory extender has not been identified). Such samples can be diluted somewhere in the cryopreservation process, for example, just prior to addition of cryoprotectant to minimize time in extender. Overall, the decision to dilute or not, and at what ratio is driven in practice by the need to hold samples for at least 24 hr. This is to enable shipping or to avoid rushing during processing, especially when work can be done most efficiently in batches rather than with single individuals.

Estimation of Sperm Concentration

This is an extremely important factor in standardizing work and ensuring quality, but it is usually overlooked or not reported. There are actually three components relevant to this factor. The first is to *estimate the concentration* by some reliable method, such as by use of a counting chamber (e.g., hemocytometer), or spectrophotometry (see chapter by Cuevas-Uribe and Tiersch in this volume, Tan et al. 2010, Dong et al. 2005a). Second is to then *adjust the concentration* to some established value. Third is to accurately *report these concentrations*. Most studies do not estimate concentration, some make estimates but do not make adjustments, and as such, these values are not reported.

From our work over the past 15 yr, there is no doubt that uncontrolled variation in sperm concentration will affect results either by directly reducing post-thaw quality, or by introducing considerable levels of uncontrolled male-to-male variation based on sperm density, not on the response to cryopreservation *per se*. It is possible for high sperm concentrations (e.g., 10^9 and above) to overwhelm the amount of cryoprotectant available. This effect is variable with the relationship between the concentration of sperm and concentration of cryoprotectant, but with a consistent result (i.e., too little cryoprotectant for the number of sperm).

Therefore, sperm concentrations should be set at an optimized value in relation to the cryoprotectant concentration. This value should take into consideration the cryoprotectant toxicity (which will limit cryoprotectant concentration), equilibration time (which should be sufficient for cryoprotectant permeation, while allowing sufficient handling time for samples, yet not be so long as to produce toxic effects), container type (chosen for ease of handling, labeling, and storage), cooling rate (which interacts with cryoprotectant and container), and practical constraints in costs, storage space, shipping capabilities (samples that are excessively dilute require extra containers and space for a given number of sperm), and use for fertilization (avoiding shortages or waste of sperm on a per-female or per-egg basis). This may seem like a lot of factors to balance, but these factors do not disappear if ignored, and as such should be directly addressed. If reliable research results and post-thaw quality are to be goals, the first step is to estimate the sperm concentration.

Motility Duration Studies

As part of the process of working with a new species, or simply assessing samples after collection or shipment, it is useful to identify the duration of motility. As stated previously, motility (especially peak motility) is often short-lived (e.g., < 30 sec). However, it is not unusual for continuous motility to last for longer periods, for example in oysters (Paniagua-Chavez et al. 1998) or live-bearing fishes such as *Xiphophorus* (Yang et al. 2006). The longer duration offers benefits in handling and makes motility assessments easier and more accurate. A straightforward experimental method to characterize motility duration involves two people. An experienced technician can activate the sperm on an uncovered slide and estimate motility at specific time

intervals, while another technician records the values and prompts the estimates at the appropriate times. This experiment can be repeated and the results graphed (Figure 1).

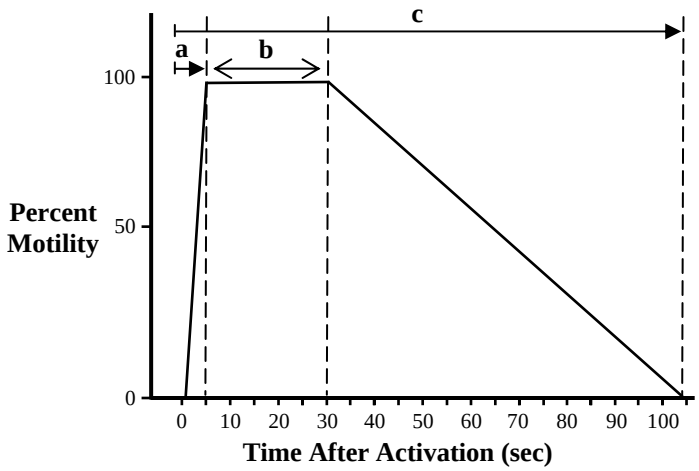

Figure 1. Diagrammatic characterization of sperm motility for a representative freshwater fish. Motility can be divided into three periods: a) time until complete motility (activation); b) duration of complete motility, and c) time until cessation of motility.

This knowledge will benefit planning of experiments by clearly indicating the timing involved for motility estimation or for mixing of gametes to ensure optimum fertilization. Of course these estimates can be made by computer-assisted sperm analysis (CASA) if the instrument can record accurate data within the necessary window of observation. The duration of motility can be expected to be different for sperm at the time of collection, after refrigerated storage, after addition of cryoprotectant, and after thawing. Thus it is valuable to repeat this experiment at those times. In addition, motility duration (and swimming speed) is often affected by osmotic pressure (see below).

Osmotic and Ionic Activation Curves

Most fish spawn by external fertilization, releasing sperm and eggs into the water. Unless certain ions are involved (such as potassium in salmonids), in freshwater species, sperm motility can usually be activated by reducing the salt concentration of the sperm solution in comparison to that of blood. In marine species, motility can be activated by increasing the salt concentration. These are general observations and can serve as a starting point for characterizing sperm motility activation (and perhaps oocyte activation as well) for a new species. It should be noted that aquatic species present a broad variety of biological mechanisms that require individual attention. For example, live-bearing fishes that utilize internal fertilization exhibit a sperm activation zone that is isotonic to body fluids – this may be initially surprising in light of what is observed for freshwater and marine species, but makes sense in that the environment which the sperm needs to be motile (i.e., the female reproductive tract) is isotonic (Figure 2, next page).

As stated above, once activated, aquatic species sperm typically have a short life span. Thus sperm need be maintained in an extender with proper salt concentration (usually nearly isotonic to the blood plasma) to inhibit undesired sperm activation during refrigerated storage or cryopreservation.

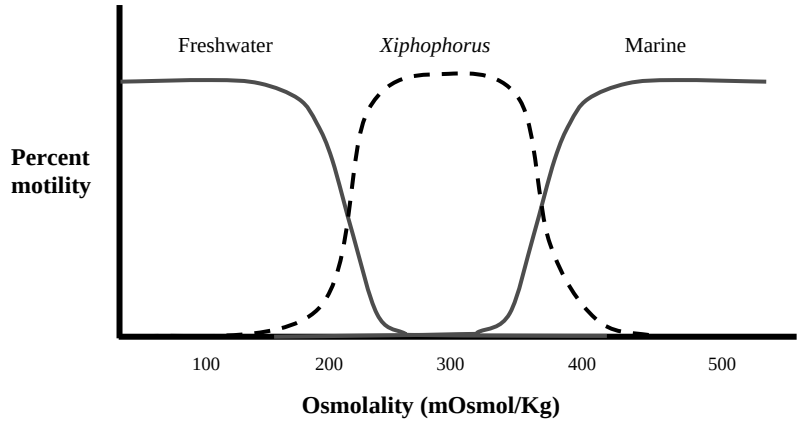

Figure 2. Some generalizations can be made about the osmotic response of sperm motility for species based on the external spawning environment or the mode of reproduction (such as for live-bearing fishes of the genus *Xiphophorus*). Generalizations however, do not substitute for specific knowledge, and motility activation should be characterized for any species being studied.

By evaluating motility across a range of osmotic pressures the basic pattern of activation can be characterized and used to make informed decisions about development of extenders necessary for maintenance of a quiescent state for storage, and the conditions necessary for complete activation of sperm to facilitate fertilization. Our approach is to expose sperm to a graded series of dilutions (spaced approximately at intervals of 30 mOsmol/Kg) of the candidate extender to identify the point of *complete activation*, defined by us as the highest osmolality that elicits the highest motility observed for the sample (this is often not 100%), and the point of *threshold activation*, defined by us in practical terms as 10% motility. A dilution of 1:20 (v:v, sperm:test solution) is typically employed to ensure that the osmotic pressure tested is correct and it is verified with an osmometer by testing a 10-uL aliquot taken directly from the microscope slide immediately after motility assessment (Figure 3).

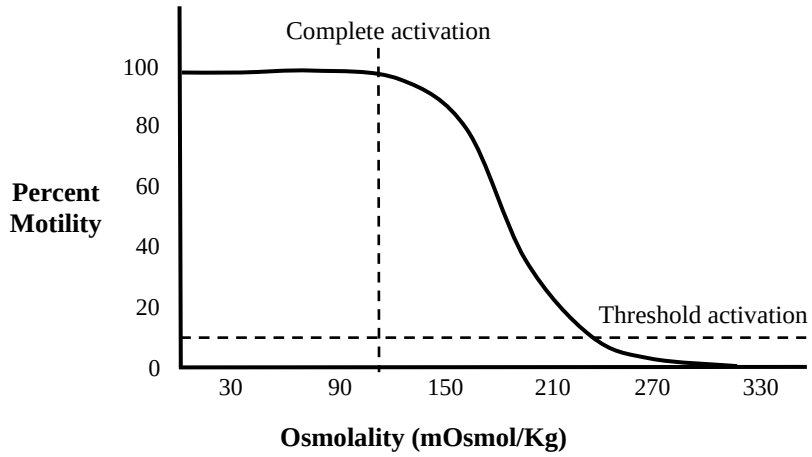

Figure 3. A representative osmotic activation curve produced at 30 mOsmol/Kg intervals for a freshwater species. In this example complete activation occurs at ~110 mOsmol/Kg and below. As such, osmolality of fertilization medium should be reduced below this value to ensure complete activation of gametes. Threshold activation occurs around 240 mOsmol/Kg. As such, extenders should be well above this value (at $\geq$ 300 mOsmol/Kg) to ensure non-activated sperm storage.

Duration and intensity of motility can be affected by osmolality as well as the pattern of activation. For example, motility will be briefest at lower osmotic pressures for a freshwater fish (unless damage occurs from exposure to hypotonic solutions), and more prolonged at higher pressures (until reaching isotonic levels) (Figure 4).

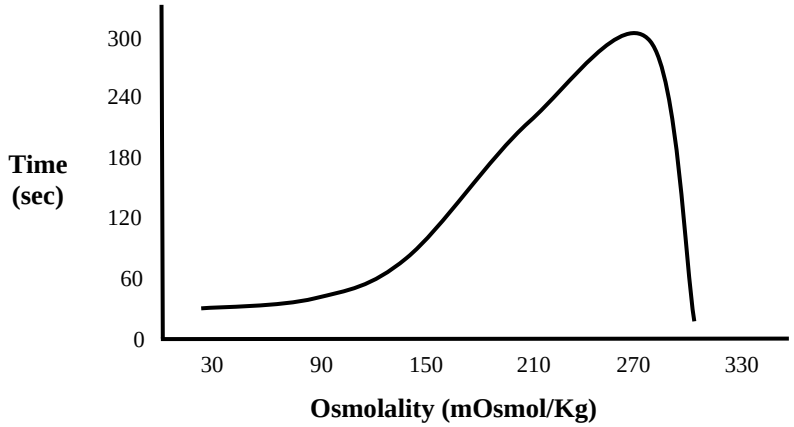

Figure 4. A representation of motility duration across a range of osmotic pressures. This relationship is sometimes used to prolong swimming time with the assumption that it will improve fertilization, but it should be tested for effectiveness in any species (and fertilization medium).

It should not be assumed that a longer swimming time will increase fertilization rates, especially in comparison to other manipulations such as minimizing the fertilization volume (see below in Fertilization Unit section), without evaluating the activation of oocytes (and fertilization) in the same medium. Moreover, a fertilization solution that is used with fresh sperm to prolong motility duration may not be effective with thawed sperm – the osmotic pressure due to the cryoprotectant (even after dilution) can be additive with the osmotic pressure of the fertilization solution and raise the overall value above that which will allow gamete activation (this will lead to the mistaken conclusion that the "cryopreservation did not work"). The activation curve thus offers valuable information to help avoid and troubleshoot problems.

Sometimes an activation curve will indicate that osmotic pressure *per se* is not the dominant factor in controlling motility activation. In these cases, it is possible that the increase or decrease of a particular ion or the presence of another molecule (e.g., egg protein) can be activating motility. This will require more research to elucidate the mechanism of motility activation, and perhaps to identify a suitable extender that allows at least 24 hr of refrigerated storage (see below). A good practical starting point to address this is to test the effect of non-ionic solutions (e.g., sugar solutions) on motility: if motility activation responds to osmotic pressure somewhat or markedly (i.e. similar to Figure 3 for a freshwater fish) it can be hypothesized that the reduction of a particular factor (e.g., a certain ion) is the activating trigger, or that it at least interacts with osmotic pressure; a lack of activation could lead to the hypothesis that the presence of a particular factor is necessary to activate motility. The effect of osmotic pressure on swimming intensity is difficult to assess by eye (beyond simple categories such as "fast" or "slow"), but is ideally suited for CASA. If possible it is best to use darkfield microscopy to evaluate motility with good accuracy and repeatability.

Refrigerated Storage Studies

As indicated above, refrigerated storage is necessary for practical work. Most importantly, it allows shipping of samples from a site such as a working hatchery to a facility that has the specialized equipment and knowledge necessary for research and performance of high quality cryopreservation. Once frozen, the samples can be stored or shipped for use at a later time. Also important is the ability to flexibly schedule cryopreservation for efficient processing in batches for steps that require the same preparation and standardization for one or multiple samples (e.g., estimation of sperm concentration as a single step for multiple samples, rather than a repeated step for individual samples). Refrigerated samples can be calmly assessed for pre-freeze quality (e.g., by CASA) and be adjusted to a optimal working concentration, with careful attention to labeling and coding to avoid the mistakes that come from rushing with samples of rapidly diminishing quality. Indeed, the capability of at least 24 hr of refrigerated storage provides the basis for a routine system of quality control and assurance for commercial-scale cryopreservation. This and the capability for overnight shipping are significant topics that have not received sufficient recognition or research attention in the published literature.

We therefore routinely try to develop refrigerated storage capability for every species that we work with. If dealing with a previously unstudied species, some "clues" should already be available to you at this point from the results of the previous motility and dilution studies described above. If the sperm behaves "typically" (e.g., as in Figure 3) it is possible to test several candidate extenders of the balanced salt buffer type. Research directed at finding an "optimal" extender (and osmotic pressure) can sometimes takes on a life of its own, and can consume considerable time. While this is not necessarily bad for a well-funded, long-term project, it would not be advisable for pilot projects, or for working with species that have short annual spawning seasons. Once again, the prevailing conditions and research goals should be kept in mind when making the decision to test additional candidate extenders or to move forward with the cryopreservation research. Again, good motility (e.g., 50-70%) at 24 hr is probably sufficient to move forward at this point in developing basic protocols (Figure 5).

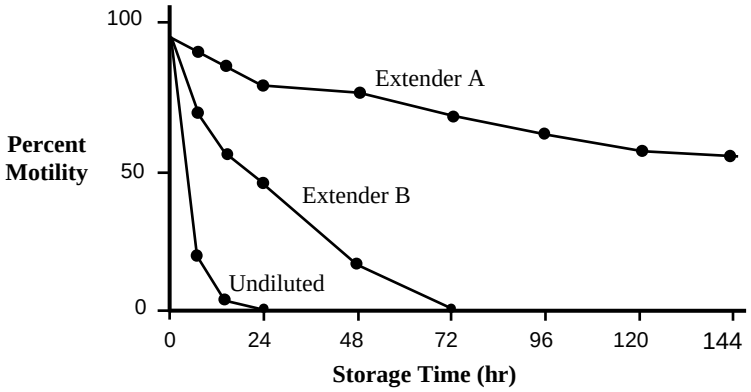

Figure 5. Representation of a refrigerated storage study comparing two extender solutions (at 300 mOsmol/Kg) and undiluted sample collected by stripping. Undiluted, stripped sperm often loses motility capability quickly compared to sperm in extender due to urine contamination. In this case, Extender B is satisfactory for routine storage and shipping (e.g., ~50% motility at 24 hr), while Extender A offers much more utility. If time or resources were in short supply, and Extender B was the best you had, you might consider using it for the cryopreservation studies that follow. The search for Extender A could wait for optimization studies.

Shipping Studies

Shipping is often a violent process. Boxes are sometimes thrown or dropped, left sitting outside in the sun or on a frozen loading dock, or opened for inspection and repackaged improperly. The point here is that storage of samples in the refrigerator for 24 hr does not constitute a realistic shipping evaluation. Practical evaluation would involve using the actual packaging that will eventually be used for samples, and sending the package overnight (even if only to yourself). Often samples are collected elsewhere and shipping is required from the beginning. In short, carefully controlled protocols that do not take the sperm outside of the laboratory may not transfer well to practical application. This could be a weak link in the protocol development pathway so it should receive consideration.

Acute Toxicity of Cryoprotectants and Equilibration Time Studies

Conventional cryopreservation involves the use of cryoprotectants and freezing rates sufficiently slow to produce cellular dehydration and shrinkage (termed “equilibrium cooling”) to avoid intracellular ice formation. Cryoprotectants are chemicals used to protect cells from damage during freezing and thawing, and are classified by whether they penetrate the cell (referred to as “permeating”) or remain outside of the cell (“non-permeating”). Although the mechanisms of action are as yet not completely understood, permeating cryoprotectants such as dimethyl sulfoxide are believed to help reduce the damage caused by formation of ice crystals within sperm cells. They also help reduce the dehydration damage that occurs when water leaves the cell to become ice in the surrounding solution. Non-permeating cryoprotectants such as sugars and polymers are believed to help stabilize the membrane during cryopreservation. Too little cryoprotectant entering the cell before cooling can reduce effectiveness, whereas too much can cause swelling and rupture during thawing and dilution. This is governed by the ability of the cryoprotectant to diffuse into and out of the cells.

In addition, cryoprotectants are often toxic to cells (including osmotic damage), and thus the choice of the types of cryoprotectant and their optimal concentration (usually a balance between cryoprotection and toxicity) has been the focus of numerous studies. After the addition to sperm samples, time is needed for the cryoprotectant to permeate the cells. This is referred to as the *equilibration time*. For most circumstances it can be set at 15 to 30 min (often on ice or at 4 ^{o}C), but it can be varied depending on the type and concentration of cryoprotectant being used, and the number of samples being processed. If the necessary concentration of cryoprotectant is toxic to the cells, the equilibration time of rapidly permeating cryoprotectants such as dimethyl sulfoxide can be shortened to the minimum time required for filling of containers (e.g., 10 min).

For practical purposes we use 15 min as our minimum equilibration time which we define as the time between addition of cryoprotectant and the samples reaching 0 ^{o}C in the freezing process. Some cryoprotectants such as methanol are relatively non-toxic, penetrate rapidly, and do not contribute to the overall osmotic pressure. Others such as dimethyl sulfoxide have some toxicity, penetrate rapidly, and contribute markedly to osmotic pressure. Other cryoprotectants such as dimethyl acetamide, are sometimes so toxic that they are not useful for some species. How can this be determined in a practical manner? We utilize acute toxicity studies (Figure 6) to narrow down the list of cryoprotectants and select the concentrations to be used in subsequent cryopreservation experiments.

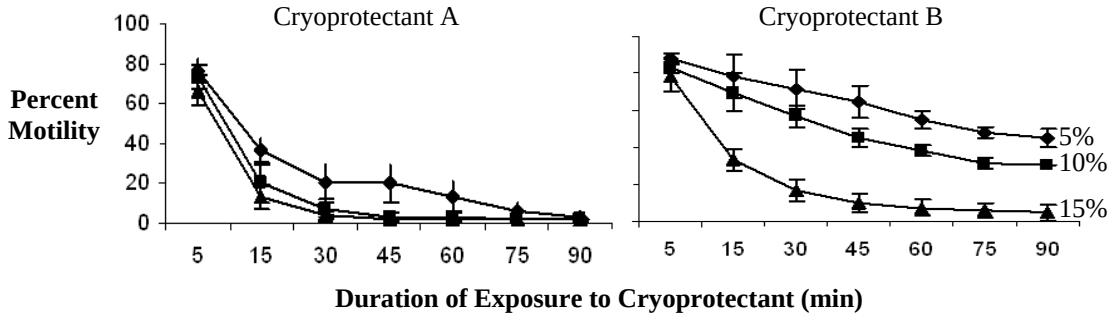

Figure 6. Representation of an acute toxicity study for two cryoprotectants at three concentrations (5, 10 and 15%). Cryoprotectant A reduces motility quickly and does not allow much time for permeation or handling. If other candidates are not available, 5% offers the least toxicity, but may not offer sufficient cryoprotection. Cryoprotectant B however has less effect on motility, and 10% would be a suitable choice for a 15-minute equilibration period. This may be a useful cryoprotectant concentration, especially if the sperm concentration is controlled.

By following this process we can reduce the number of cryoprotectants and concentrations to be tested, greatly speeding the research process. The reasoning behind this is that if the sperm cells are damaged (immotile) by cryoprotectant toxicity before they are cooled, they will not be resurrected by the ensuing freezing and thawing ("do not kill the cells before you freeze them"). We use sufficient dilution to ensure that the osmolality of the aliquots tested is appropriate for motility activation. This is verified by testing the actual sample from the microscope slide with an osmometer (i.e., the loss of motility is not due to an osmotic constraint on activation).

The pursuit of "optimal" cryoprotectants and concentrations can take on a life of its own. Indeed, it is not uncommon for problems in other aspects of the cryopreservation process to be incorrectly ascribed to cryoprotectant selection, and an empirical survey undertaken to test whatever chemicals that are at hand or have been recently been published with exciting results. I view this as a trap and would suggest that practical protocols for the vast majority of aquatic species can be developed using a small number of cryoprotectants such as methanol or dimethyl sulfoxide, and secondarily glycerol or dimethyl acetamide, perhaps in combination with specific sugars. In any event, these should be the starting points in a strong research pathway controlling the major variables. Pursuit of exotic cryoprotectants has a place as an optimization step, or for those recalcitrant species that pose special challenges.

Equilibration Motility

Given, as explained above, that cryoprotectant toxicity can produce non-viable cells prior to freezing, it is essential to be able to identify this effect and not confuse it with the effects of freezing and thawing. For this reason *it is essential* to retain a small sample of the material being frozen and to *always* estimate the motility at the end of the equilibration period as the samples are being cooled. We refer to this as the *equilibration motility* and it is an extremely important piece of information that is not typically collected or reported. Experiments are often incorrectly interpreted as "the cryopreservation did not work" because sperm were immotile after thawing. Without a pre-freeze assessment of equilibration motility it is not possible to know how much of the motility loss came before cryopreservation. It is important to note in this regard that samples can become more sensitive to the toxic effects of cryoprotectants during refrigerated storage or

shipping, and thus samples that initially showed little effect from 10% cryoprotectant could suffer dramatic drops in equilibration motility after 24 or 48 hr. Therefore, as stated above, *always* estimate motility at the end of the equilibration period as the samples are being cooled.

Packaging and Cooling Rate Studies

Proper packaging of cryopreserved samples is important for numerous reasons including standardization of rates for freezing and thawing, efficient use of space for storage and shipment, assurance of permanent sample identification, proper sealing for biosecurity and safety (leaky containers can explode on thawing), and enablement of automated sample processing. Various containers have been used for fish sperm, including drinking straws, glass capillary tubes, and plastic cryovials. Plastic French straws have been in use for livestock sperm for decades. These straws come in 0.5- and 0.25-mL volumes, and are produced in over 20 colors. The use of these containers offers the advantages of efficient and reliable sample identification by permanent printing on colored straws, sample safety by complete sealing, standardization of the cooling and thawing processes because of their thermal properties and large surface area in relation to their volume, and their being designed for use with automated processing equipment. Adoption of the French straw for aquatic species has been limited by a number of factors including the notion that their volume is too small for routine hatchery use (and the idea that "free" straws are available from fast-food restaurants). However when sperm numbers are controlled, especially in relation to egg numbers, this need not be a realistic problem, and the benefits of this packaging greatly outweigh the inconvenience of handling multiple straws.

The choice of cooling rate has been another major focus of numerous studies of sperm cryopreservation. To be considered as optimal, a rate should be slow enough to minimize the amount of ice crystals that form within the sperm cells (below a damaging level) and yet be rapid enough to minimize the length of time cells are exposed to what is referred to as the "solution effect", which is the concentration and precipitation of materials that occurs when solubility limits are exceeded during the dehydration caused by ice formation. There are a number of methods that can be used for freezing. These range from the use of expensive computer-controlled freezers which offer precise and reproducible rates, to simpler and cheaper freezing by suspending samples above liquid nitrogen in a styrofoam cooler, although this is a less reproducible approach. Samples can also be suspended in the neck of a storage dewar. With these latter two methods, the height of the samples above the liquid nitrogen offers control of the temperature and cooling rate. There is no current accepted definition of cooling rate for aquatic species and it is possible that the rates reported are obtained by different means for every study. We typically use the time required *for the sample* to traverse from 0 ^{o}C to -80 ^{o}C to calculate cooling rates. Care should be taken to carefully report the method used to calculate the rate.

As indicated above for other parameters, the pursuit of "optimal" cooling rates can take on a life of its own. Because this is an important cryobiological parameter, specialists (or novices) will sometimes focus on this component at the expense of the other activities outlined in this chapter. The concept of "optimal" is extremely relative for cooling rates because they are so dependent on the choice of container and cryoprotectant. This is one of the reasons why studies seem to disagree on the relative effectiveness of specific protocols even within the same species. Sperm cells are not tremendously different in basic biophysical properties when surveyed across aquatic species, and there is no theoretical reason why each species should *a priori* be considered to be distinct from every other. A lack of confidence in the research area (especially for novices) may lead to hasty conclusions to accept a particular cooling rate or cryoprotectant as being

ineffective because of the mistaken idea that there are unique, optimal conditions awaiting discovery (the "eureka" moment). This may be responsible for adoption of the empirical survey approach often seen in aquatic species research (i.e., trying everything on the shelf) instead of addressing the more useful question of *why* a perfectly reasonable cooling rate or cryoprotectant such as methanol was not effective.

The cooling rate should be measured directly within the container being frozen. This is because large volumes or thick containers will cool at a different rate than small, thin containers when exposed to the exact same freezing conditions. How can cooling rate be tested in a practical manner? Because the factors interact with one another it is important to establish some of them from the beginning. We use plastic straws (0.25 or 0.5 mL) because of their numerous advantages and their capability for automated processing with enables high-throughput approaches. With the container chosen, we proceed to evaluate selected cryoprotectants and concentrations (described above) at specific cooling rates. If time or resources are in short supply we will initially test two rates: 4 ^{o}C ("slow") and 40 ^{o}C ("fast"). Based on these results we will then focus in on the range of cooling rates to be tested or change other parameters (Figure 7).

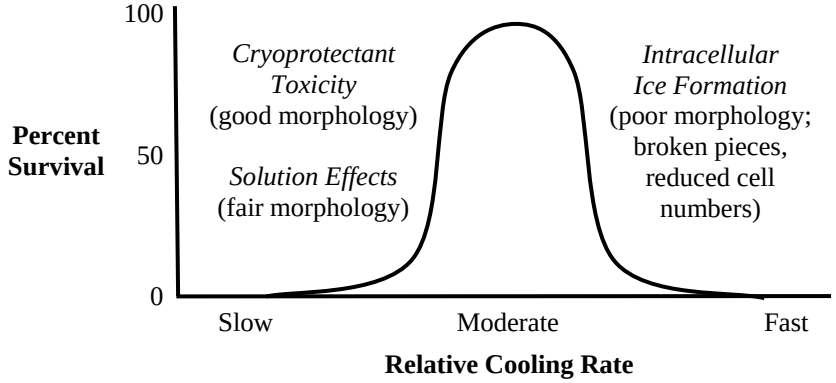

Figure 7. Diagrammatic representation of how to use post-thaw assessment to identify problems in a cryopreservation protocol. Basically, if the cells are intact ("beautiful corpses") but immotile (dead), they were likely victims of cryoprotectant toxicity (check the equilibration motility). If they have fair morphology but little survival they might have been frozen at too slow of a rate and succumbed to the effects of high salt concentration or low pH (e.g., protein denaturation). If there are few cells visible and the background is littered with sperm fragments, they likely were frozen too fast (or had too little cryoprotectant, or the cell concentration was too high) and were destroyed by ice formation. This information can be used to make adjustments in subsequent experiments.

After this initial assessment, we can narrow experiments down to a few cryoprotectants (at a single concentration) and if necessary a reduced range of cooling rates. The interval between rates tested and the reproducibility of the profiles is dictated by the freezing method. We routinely use a computer-controlled freezer to obtain accurate and reproducible rates. This is not essential however, and reasonably reliable cooling can be obtained by a variety of methods including styrofoam boxes, or even dry ice, if proper safeguards and protocols are used. Regardless of the method used, the reasoning behind research decisions will remain the same. For example, cryoprotectants can interact with cooling rate. Certain cryoprotectants may function better or worse than others at specific rates under the conditions being used, and it is possible to inadvertently test outside of the effective rates for specific cryoprotectants (Figure 8).

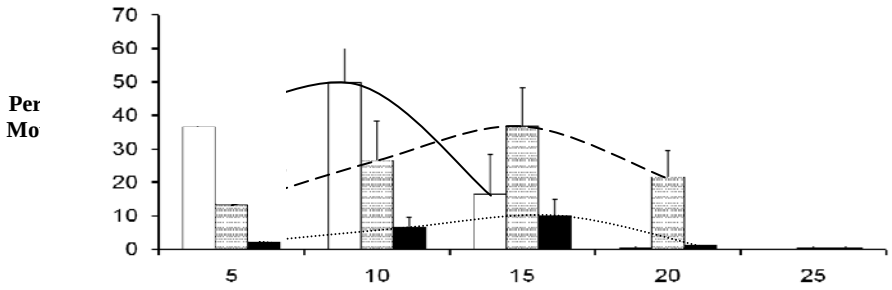

Figure 8. A representative comparison of three cryoprotectants at five cooling rates. Cryoprotectant A (white bars) performed well at the slower rates (best at 10 ^{o}C/min). Cryoprotectants B (gray) and C (black) performed better at faster rates (best at 15 ^{o}C/min), although cryoprotectant C never achieved the same performance as the other cryoprotectants. From this we can conclude that there is no single "optimal" cooling rate, and that cryoprotectants do not have equal protective abilities. In addition, note the abrupt drop at the 25 ^{o}C/min rate for all cryoprotectants. Without careful control of cooling rate, or examination of lower rates, it could be incorrectly concluded that none of these cryoprotectants were effective, and that empirical testing of new, exciting cryoprotectants was necessary.

As stated above, cooling rates should be monitored within a container (preferably within a sample of the actual liquid being frozen) by use of a thermocouple and recorder. Cooling rate should be reported along with the container type, and if possible, the rate or temperature within the chamber (freezer environment) along with the programmed rate (if using a controlled-rate freezer). Also be aware that numerous factors will affect cooling rates including: the ambient room temperature, the number of containers being frozen at one time (thermal mass), whether freezing cycles are being repeated (successive runs can be different), whether frost has accumulated within the freezer (a problem in humid environments like Louisiana), low levels of liquid nitrogen in the pressure tank that drives the controlled-rate freezer, and so on. This list alone provides ample additional reasons why the actual rate within a container should be measured during every cooling cycle.

Thawing Rate Studies

In simple terms, freezing and thawing each present a "danger zone" to cells as they traverse between the temperatures of 0 ^{o}C and -40 ^{o}C. As such, thawing can be as destructive to cells (albeit for somewhat different reasons) as cooling. In general, rapid thawing is preferred to minimize the damage associated with recrystalization (the coalescence of small ice crystals into large crystals during thawing). Samples should be removed from the storage dewar and transferred immediately to a container such as a styrofoam box that can safely hold liquid nitrogen. This ensures that samples will not thaw prematurely due to handling. For our work, straws are held in a 20 to 40 °C water bath (a thermos or small ice chest will also work) for 7-30 sec. Specific times and temperatures should to be optimized for the particular sample volume, container type (e.g., volume, materials, and geometry all affect the rate of heating), and species (e.g., cold water or warmwater fishes). We sometimes test a range of thawing temperatures (e.g., from 0 to 60 °C) to optimize protocols for a variety of species. As a rule of thumb, samples can be considered to be thawed when air bubbles within the container can move freely within the liquid. The use of transparent or translucent containers will aid in viewing the sample. The

samples should be cool to the touch when thawed (not warm), or even cold when working with coldwater species such as salmonids. For research, motility should be estimated *immediately* after thawing. This is because sperm can sometimes be motile in the container after thawing (without addition of activating solution) and this motility can be lost quickly (i.e., < 15 sec, unpublished observations). As such it also follows that fertilization should be tested immediately after thawing unless experiments have been done to determine the useful lifetime of post-thaw samples (this could be tested as part of the application pathway). On the other hand, when using relatively non-toxic cryoprotectants such as methanol, post-thaw sperm can sometimes be refrigerated for days with satisfactory motility (e.g., Tiersch et al. 1994).

Post-thaw Amendment Studies

Sometimes depending on the species or application it is necessary to evaluate treatment of the sperm after thawing to improve performance. Usually it is sufficient to thaw the samples, place them on eggs, and add water to activate the gametes. For live-bearing fishes of the genus *Xiphophorus*, however, thawed sperm requires washing to remove cryoprotectant, and concentration of the cells (to achieve an appropriate sperm density) and to reduce the sample volume for artificial insemination into the female reproductive tract. To accomplish this, studies were necessary to identify the conditions for proper centrifugation and resuspension of the pellet (e.g., Dong et al. 2006).

Post-thaw Quality Assessment

As stated above, qualitative observations of thawed sperm can be quite helpful in evaluation and troubleshooting of protocols. For example, to review, if the sperm cells are visibly damaged, the cryoprotectant concentration may have been too low, the cell concentration too high, or the cooling rate may have been too rapid. Conversely, if the cell morphology is intact although the sperm are immotile, the concentration of the cryoprotectant may have been too high; this would be confirmed by a reduction in the equilibration motility observed before freezing. Assessment therefore must extend beyond the simple, often incorrect, observation that cryopreservation did or did not “work” (an observation heard countless times).

By considering all of the steps involved the entire process pathway, verifying quality and controlling variables at each step, using screening to narrow down the number of variables in successive experiments, and developing a basic understanding of cryobiological theory, it is possible to evaluate thawed samples and make informed decisions about the problems in the overall process. Perhaps there is a single step that accounts for substantial motility loss, or small quality losses that are accumulating across a number of steps. It can be as simple as looking into the microscope and recognizing that the background is gray, not black as it should be in darkfield microscopy. If the scope is set up properly, this usually indicates that the cells have been destroyed by intracellular ice formation and the cooling rate was too fast (assuming the cryoprotectant was sufficient). In other words, within 1 sec of viewing and without seeing a single sperm cell it is possible to begin to diagnose problems (yes, it can be that simple).

If possible, it would be best to evaluate sperm with a battery of tests such as those characterizing membrane and nuclear integrity, mitochondrial potential (e.g., flow cytometry), motility, and fertilization capacity. This would present a more complete picture of how and where damage has occurred. Even apparently simple cells such as sperm have a variety of compartments that can respond differentially to a set of cryopreservation conditions and would be best characterized by a combination of quality assessment assays (Figure 9).

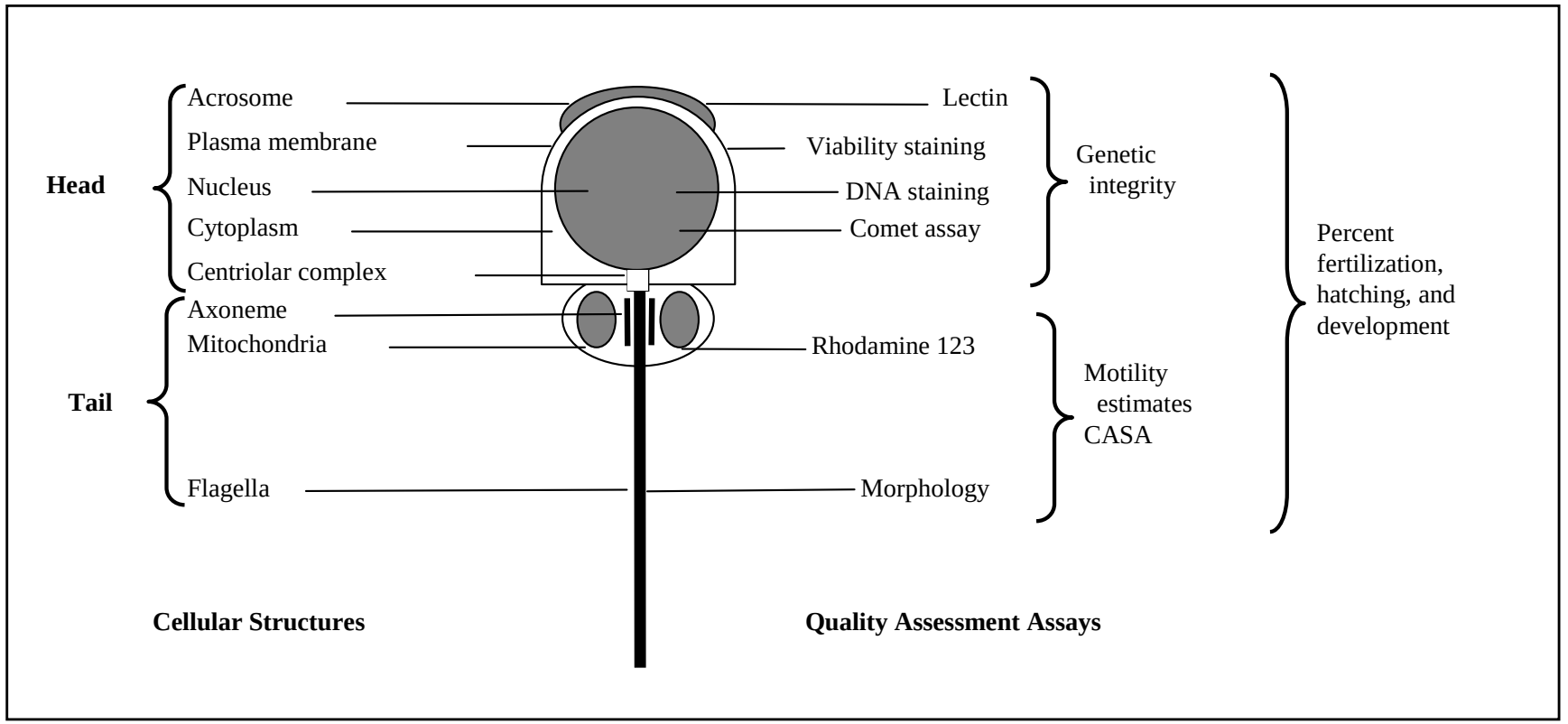

Figure 9. Sperm quality is a generic term that encompasses proper functioning of a combination of cellular structures (indicated on the left of the sperm cell) that can be evaluated individually by specific assays (indicated on the right) or in aggregate by examining factors such as the capacity of sperm to fertilize eggs that hatch and develop normally. Sperm quality can be affected by a number of factors in cryopreservation process pathways. Indeed, damage can occur to specific structures (for example the acrosome or nucleus) that would not be detectable by study using a single assay (such as motility). Assays such as these have been applied in aquatic species, but an integrated battery of assays that would encompass a comprehensive analysis of sperm quality does not yet exist.

An example of the compartmentalization of damage possible for sperm cells during cryopreservation is the gelling of post-thaw samples seen in Pacific oysters *Crassostrea gigas* (e.g., Dong et al. 2007a). Sub-optimal conditions (e.g., cooling rate, cryoprotectant concentration, or cell concentration) can cause damage or activation of the acrosome. This causes the cells to form aggregates, reducing fertilization capacity despite otherwise acceptable cell morphology. For practical purposes in the research pathway, we generally use a 50% recovery of viable (motile) sperm (based on the original starting point) as an acceptable goal.

Fertilization Assay Studies

Artificial spawning involves the collection from females of unfertilized eggs to be combined with sperm. Unless testing has shown otherwise, it is generally best to minimize the time between thawing of sperm and fertilization. After thawing, sperm samples are added to eggs and thoroughly mixed, and the gametes are activated with an appropriate solution (this is called the "dry method"). Other fertilization methods are available and can be evaluated for use with any particular species. Fresh sperm samples can be used to fertilize replicate batches of eggs to serve as a test for egg quality (unless factors such as sperm numbers are controlled it is generally not statistically appropriate to make direct comparison of the fertilization produced by fresh and thawed samples). It is important to quantify the fertilizing ability of each batch of eggs because variation in egg quality across females can be considerable. It is likewise advisable to replicate all treatments (e.g., fresh and thawed from each male) that will be statistically compared with eggs from each female (e.g., the female represents a replicate) and to establish *a priori* the minimum egg quality to be included in statistical analysis (e.g., at least 50% fertilization with fresh sperm). If the females are small and only produce a few eggs (such as medaka *Oryzias latipes* or zebrafish *Danio rerio*) pooled batches from several females can be used to obtain the requisite number of eggs (e.g., Yang et al. 2010). After ~5 min, additional water is added to water-harden the eggs. Percent fertilization can be determined to evaluate sperm quality.

Artificial spawning in this way allows for a variety of crosses such as the use of one male to fertilize eggs from several females, or for the eggs of one female to be fertilized by sperm from several males. This can lead to a breeding matrix where a group of select males can be mated with a group of select females to develop populations with distinctive traits. By having assayed the parents for genetic markers, breeders can develop broodstocks with enhanced characteristics, such as growth rate or disease resistance. Subsequent gene mapping can be used to further enhance and balance desirable phenotypes. Such a process can also be used to cross two different species to develop hybrids with improved traits, although precautions should be taken to prevent the accidental escape of the hybrid fish into the wild.

There is no current accepted definition of percent fertilization. Approaches vary considerably among studies ranging from first mitotic division to hatching and beyond, using absolute values or values relative to controls. Thus it is problematic or impossible to directly compare fertilization values among studies.Care should be given to report clearly the criteria used. In addition, it is advisable to choose a life stage (e.g., neurula) sufficiently developed that it represents post-zygotic activity rather than oocytic activity, yet not so late in development that culture conditions or other non-tested factors could reduce survival in a way that could be confounded with treatment effects.

Establishment of Optimal Dilutions and Concentrations

Estimates of sperm concentration can be made by a number of methods (see chapter by Cuevas-Uribe and Tiersch in this volume), and are essential to collect reliable data necessary to develop protocols (Dong et al. 2007a). In addition, estimation and adjustment of sperm concentration provide the foundation upon which quality assurance and optimization are built. As such, cell concentration should be assessed early in the pathway (described above). This method is vastly superior to simple standardized dilutions of samples. Nonetheless, dilutions are necessary in the pathway (e.g., at sample collection, when adding cryoprotectants, or for fertilization after thawing), and when dilutions are based on an initial sperm concentration they simply become sperm density adjustments.

It is at this stage in the process that some optimization is advisable for sperm concentrations and the dilutions necessary to produce appropriate volumes. Some variables of importance are the concentration and total number of sperm per straw (e.g., can be used to maximize storage efficiency), the sperm numbers (total or percent motile) in relation to the egg numbers (e.g., can be used to match the cryopreservation unit – such as a straw – to the number of eggs available per spawn), the total volume used for individual fertilizations (e.g., it is often useful to minimize this volume to enhance sperm-egg interaction), and the sperm-to egg-ratio (e.g., it can be used to avoid waste of excess gametes, and to standardize assay conditions for direct comparison of treatments). Practical protocols and pathways will pay close attention to optimizing these volumes, dilutions, and concentrations *with respect to overall efficiency*, not just enhancement of any single step.

Calculation of the "Fertilization Unit"

As part of this process it is useful to perform the calculations necessary to match your protocol to how it will be eventually be used. Large-boded fishes that produce hundreds of mL of diluted sperm will need different calculations than will small-bodied aquarium species that produce microliter volumes (Tiersch 2001), and fertilizations used to mass produce offspring in a commercial foodfish hatchery will need different calculations than a conservation program aimed at producing numerous separate families to achieve proscribed levels of genetic diversity. These calculations often will need to strike a balance between the expected levels of gamete quality and the desired numbers of offspring.

This is where research protocols leave the sheltered environment of the laboratory and have to earn a living in the cold, hard world. The earlier in the process that consideration is given to specific forms and levels of application, the more useful the protocol will likely be. For example, it is not efficient to do research with a container that is not suitable for hatchery application, because large amounts of the work will have to be repeated to re-establish the necessary working conditions (e.g., cooling rate, possibly cryoprotectant and concentration, and batching methods). One approach we use is to calculate backwards from the numbers of sperm and eggs needed, and to base the cryopreservation container on the expected number of eggs. For example, calculations could be performed to establish one straw per female as the basic "Fertilization Unit" (Table 2).

Table 2. Sample calculations used to establish the "Fertilization Unit" for zebrafish sperm collected by two methods based on the use of a single 250-ul straw and the eggs (~100) from a single female.

Step in Process	Sample Type	Sample Volume	Concentration (sperm/ml)	Sperm Number	Sperm Number Definition
1) Sperm collection	Testis	4 µL (4 mg)	1.0E+10	4.0E+07	Total # sperm available
	Stripped	2 µL	1.0E+10	2.0E+07	Total # sperm available
2) Initial dilution (1 in 200)	Testis	800 µL	5.0E+07	4.0E+07	Total # sperm available
	Stripped	400 µL	5.0E+07	2.0E+07	Total # sperm available
3) Add cryoprotectant (1 in 2 dilution)	Testis	1600 µL	2.5E+07	4.0E+07	Total # sperm available
	Stripped	800 µL	2.5E+07	2.0E+07	Total # sperm available
4) Load in 250-µL straws	Testis	200 µL (x 8)	2.5E+07	5.0E+06	Sperm in single straw
	Stripped	200 µL (x 4)	2.5E+07	5.0E+06	Sperm in single straw
5) Fertilize 100 eggs (+300 mL activating solution)	Testis	500 µL	1.0E+07	5.0E+06	Sperm in single fertilization
	Stripped	500 µL	1.0E+07	5.0E+06	Sperm in single fertilization

An Application Pathway (Protocol Utilization)

By this time, a fairly robust process pathway would be in place to provide a base for application. Many of the research pathway steps could be retained to ensure quality control, other factors could benefit from optimizations relevant to the specific application, and new research topics could become relevant. Emphasis here should be placed on improvement of overall integration and efficiency. Relevant questions include: who will apply the technology and use the samples, and how will they use them? What will be the scale of the application, and will it function as a self-contained entity, or be part of a larger enterprise? How much will it cost in terms of facilities, equipment, personnel, and supplies (e.g., Caffey and Tiersch 2000)? Does ownership (e.g., of the genetic resources, or the samples themselves) change as samples move through the pathway, and who will derive benefits from the process and products? As such, it is useful to look at an overview of the activities that would be involved (Figure 10).

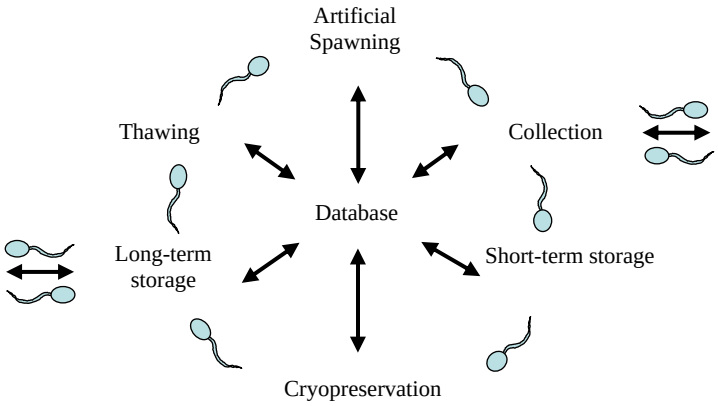

Figure 10. Representative minimal activities necessary for application of sperm cryopreservation at a single location (linked in a circle). Consecutive components are shown as a clockwise flow of sperm, and two-way arrows are used to indicate maintenance of a centralized database for information on biological characteristics, motility assessments, quality control points (shown as sperm cells), fertilization, and inventory control. Refrigerated (right side) and frozen sperm (left) can enter and leave the facility necessitating establishment of a biosecurity program (Caffey and Tiersch 2000).

For the sake of brevity only new topics in the application pathway (identified in Table 1) will be addressed below. Topics previously addressed above, such as assessment of motility at time of collection, after equilibration, and immediately after thawing, and control of sperm concentration at various points although not listed below *remain essential for the application pathway* and should not be disregarded.

Improvement of Male Quality

This is included as an example of a new research topic, and as an example of how the application pathway involves extension beyond the narrow confines of the research pathway. This might include study of control of the broodstock conditioning environment (e.g., photoperiod and temperature), hormonal induction of gonadal or gametic development, dietary manipulations to improve gamete quality, quantity, or freezing resistance, or improved husbandry to maintain family or age groupings. It could also involve multigenerational record-keeping to identify specific individuals or lines that bear advantageous alleles or gene combinations.

Shipping Studies

As indicated above, it is often necessary to ship or transport refrigerated and frozen samples. This becomes even more important when developing an application pathway. First there is the need to protect against or be able to assess damaging or wasteful accidents. Even dewars expressly designed for shipping can be dropped or stored upside down (this greatly reduces the cryogenic storage time). Thus, for valuable samples, a temperature data logger or a thermometer that records maximum and minimum temperatures should be included within the shipping container. We have had several very strong plastic cases shattered during overnight shipping (although the samples remained intact), and have even had shipping dewars arrive with the factory vacuum seal ("bung") smashed (because of the loss of the insulative properties of the vacuum, these samples were thawed and ruined). Next would come the need to achieve efficiency in transport which can come from revisiting the fertilization unit calulations and perhaps manipulating the numbers of sperm per straw. Remember, a change from 10^9 sperm per straw to 10^8 would require ten times the number of straws to transport the same number of sperm. This can quickly add up beyond current capabilities (e.g., the number of shipping dewars that are available) or drive up shipping costs beyond acceptable levels (Hu et al. 2011)

Labeling, Packaging, Storage, Inventory, and Database

Proper labeling of cryopreserved samples is essential for application. Samples can be in storage for weeks, months, or years before they are thawed. Improperly labeled samples can cause delays in processing, and even worse, could cause genetic contamination of pure stocks. The necessity for proper labeling cannot be overemphasized. *The value of samples is directly proportional to the quality of labeling information and record keeping*. Unlabeled or poorly labeled samples are essentially worthless and can even be detrimental. At the minimum, containers should be permanently labeled to indicate species, male identification number, date and facility. With the use of straws and automated processing more sophisticated labeling, such as individualized printing of alphanumeric character strings and bar coding are possible. Containers intended for archiving and breeding uses should receive the best labeling possible in conjunction with establishment of robust databases. A powerful interactive database is being established by the USDA National Animal Germplasm Program for its archival collections (it

can be viewed at: *www.ars.usda.gov/Main/docs.htm?docid=16979*). Maintenance of a current and accurate inventory can become extremely demanding and costly as a consequence of successful application and scaling up of cryopreservation. It is advisable to plan ahead for this. It can also require development of or linkage to uniform coding systems for sample identification (e.g., classification by genetic markers).

Growth and Survival Studies

Studies of this sort will become valuable to evaluate any possible effects of cryoprotectants or cryopreservation on offspring, and to keep track of the phenotypes of the animals produced by the specific matings that cryopreservation can make possible. This could include monitoring of genetic progress in agricultural settings or verification of specific gene mutations or transgenes in reconstituted research lines.

Quality Control Points

As introduced above, it is important to note with respect to cryobiology that sperm have a high degree of internal complexity. The various structures within a sperm cell represent different functional compartments that can each require different optimal conditions, and thus can each exhibit differential responses to cryopreservation. This can cause a variety of damages and outcomes. For example, damage to the tail could interfere with motility whereas damage to the head could interfere with embryonic development. Sperm quality is a generic term that encompasses proper function of a combination of cellular structures (such as the head, membrane, and tail) that can be superficially evaluated individually by specific assays, or in aggregate by examining factors such as the capacity of sperm to fertilize eggs that hatch and develop normally. For this reason it is important to evaluate the quality of thawed sperm by fertilizing eggs and monitoring the development of the offspring, which can be expensive and difficult. This can sometimes be accomplished by use of a surrogate species to provide eggs when the sperm comes from a species that has valuable or limited broodstock, or has a restricted spawning period. Hopefully at some point it would be possible to establish reliable predictive assays of fertilization capacity, such as by CASA or flow cytometry, to standardize and streamline the process.

With development of an application pathway, it is possible to incorporate regular quality control points into the process (e.g., Figure 10). A balance is necessary for the research desire to collect as much information as possible and the practical constraints of time and expense that would dictate collection of data at a few key points along the pathway. These points could be determined on a species or application basis.

Biosecurity Protocols

The collection and transfer of gametes from aquatic species poses concerns for unintended transfer of microbial organisms (Tiersch and Jenkins 2003). Sampling could come from infected broodstock (especially if they have immunosuppression arising from the stress associated with capture, transport, or handling for spawning). Contamination of samples can occur during collection, processing, storage, and transport. Generally, proper sanitation during collection is essential for limiting the spread and growth of microorganisms such as bacteria, viruses, fungi, mycoplasmas, and parasites. Materials and equipment used to freeze samples should be sterile. Establishing and following good practice guidelines for handling and processing of samples is especially important for wild-caught animals where disease-free status

cannot be guaranteed. The research and application communities and stakeholders should initiate a process to develop guiding principles or best practices for this very important topic.

A Commercialization Pathway (Programmatic Development)

This pathway brings us to *terra incognita* for aquatic species cryopreservation and exploitation of genetic resources and consequently the following statements will be brief (which potentially understates their importance). As stated above, programmatic development and commercialization are not yet available for fish and shellfish. Programmatic development can proceed along a large number of paths, but would probably proceed with private sector involvement first utilizing cryopreservation technology to improve current practices and ultimately developing into an industry based on such things as genetic resources and the provision of products (e.g., frozen sperm for increases in hatchery efficiency), genetic value (e.g., germplasm from elite broodstock) technology services (e.g., cryopreservation for hire), training, and information services (e.g., tracking genetic improvement across a given industry such as catfish or oysters). Activities of this sort have developed over the past century for genetic resources of livestock species (Caffey and Tiersch 2000) (Figure 11).

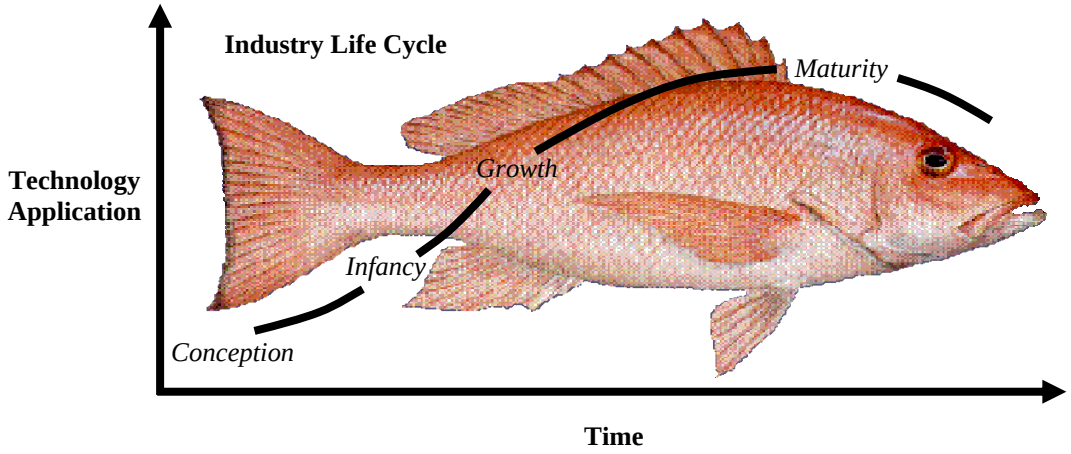

Figure 11. Diagrammatic representation of a prototypical industry life cycle. Industrial development in genetic resources of aquatic species could parallel that experienced by livestock commodities during the 20th century.

Scaling-up for High Throughput, and Establishment of Commercial-scale Facilities

The initial barrier for industrial or programmatic development will be in establishment of the facilities, equipment, and protocols necessary to produce the volume of materials and activities necessary to enable widespread adoption of cryopreservation technology, services and products. The research necessary for scaling up from the laboratory is only beginning in aquatic species, but has been recognized by groups such as the US National Institutes of Health in a recent workshop entitled *Achieving High-Throughput Repositories for Biomedical Germplasm Preservation* (NIH 2007).

Gamete Quality Assessment and Quality Control and Assurance Programs

Quality assessment will assume a much greater role with programmatic development. Rapid and reliable methods will be needed to establish industry standards. Livestock industries

have established minimum requirements promulgated by groups such as Certified Semen Services (CSS 2011), a subsidiary of the National Association of Animal Breeders (*www.naab-css.org/*). Linkage with existing organizations such as this, or development of new organizations created specifically for aquatic species should be considered.

Standardization and Harmonization

These are necessary processes that are only beginning for research–level activities (e.g., for particular equipment or processes) in aquatic species. With forethought these research-oriented approaches could be formulated to be compatible with the needs for subsequent commercial and programmatic development, and for promulgation as industry standards.

Establishment of Markets, Valuation, and Pricing Schedules

Economic research is very much in need for the products and services associated with cryopreservation, as well as for valuation of genetic resources themselves. Extremely basic questions to be addressed include: how much is a 10% improvement in growth worth when it can be purchased in the form of frozen sperm in a straw (see chapter by Boever et al. in this volume)? How much is frozen sperm from a threatened or endangered species worth? For that matter, what is the value of frozen sperm from an extinct species? More mundane questions would address the pricing schedules for batched sperm frozen from commercially relevant males in comparison to that from elite individual high-performance males. What kind of market structures will emerge from this technology? Will they be contained within single commodities (e.g., catfish or salmon), or be grouped by region, or some other consideration such as habitat (e.g., marine or freshwater), sector (e.g., private, governmental, non-governmental organization), or application community (e.g., biomedical research, conservation programs, aquaculture)?

Development of Equipment, Supplies, and Reagents Specialized for Aquatic Species

Currently work has addressed the feasibility of working with existing livestock cryopreservation facilities for use with aquatic species (e.g., Caffey and Tiersch 2000, Lang et al. 2003, Dong et al. 2005b, Dong et al. 2007b). Newer work is addressing adoption of equipment developed for use with mammals for dedicated use with aquatic species (e.g., Hu et al. 2011). With programmatic development in aquatic species, greater need and opportunities will exist to encourage continued innovation in creation and marketing of equipment, supplies and reagents specialized for use with aquatic species.

Treaties, Regulatory Frameworks, Material Transfers, and Access and Benefit Sharing

Large-scale transfer of samples, and more importantly the genetic resources contained within, will open expansive areas of legal and ethical research to address proper approaches and protections for the new capabilities created by the availability of commercial-scale cryopreservation. This will undoubtedly involve international agreements and frameworks to promote access, enable benefit sharing, avoid inequities, and to protect investments and encourage innovation (some of these topics and addressed in this volume in an annotated original chapter by Jenkins, and a new chapter by Long and Blackburn). Existing and pending regulatory frameworks for industries such as livestock or concerns for preservation of biodiversity could supercede those that would be developed by choice within the aquatic community. Potential stakeholders should initiate development of appropriate structures and look to development of

harmonization among the network of regulatory frameworks that could come into play for global commercialization of aquatic genetic resources.

Summary

This chapter is intended to serve as an introduction to the concepts necessary to view cryopreservation research and application in a comprehensive and integrated manner. Fifty yr of previous study of aquatic species cryopreservation has not yet led to commercial application and programmatic development (despite need for them). This is due to a number of reasons including aquatic market structures, breeding and husbandry practices, and a weak base for application due to a narrow and disjointed research focus that emphasizes certain cryobiological parameters over development of balanced and functional pathways. The important conceptual message from this chapter is to promote a larger comprehensive approach for aquatic species, to treat this process as a series of pathways (rather than as independent steps) and to seek integration and balance across the steps necessary for applied goals. Significant contributions to this approach could be realized using technology transfer from established livestock industries.

Acknowledgments

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Research Methods for Cryopreservation of Sperm

William R. Wayman and Terrence R. Tiersch

In this section, we have chosen to provide a single integrated set of methods as a starting point for development of protocols and standardization of reports. These methods have been adapted for sperm cryopreservation of more than 30 species of marine and freshwater fishes in our laboratory. The information provided here can be supplemented with methods and information in other chapters to develop new protocols or to work with previously unstudied fishes. There is also a large amount of practical information available for topics such as cryopreservation and freeze-drying methods (Simione and Brown 1991, Day and McClellan 1995), cryopreservation of algae (Morris 1981) and semen of boars (Johnson and Larsson 1985, Johnson and Rath 1991). Sources such as these can be valuable for developing and using cryopreservation protocols even though they are not intended to address aquatic species. Overall, there are several basic components involved in development of cryopreservation protocols. These will be covered individually in the following sections: 1) Sperm collection; 2) Motility estimation; 3) Extenders and refrigerated storage; 4) Labeling of straws, goblets and canes; 5) Addition of cryoprotectants; 6) Filling of straws; 7) Freezing procedures; 8) Storage procedures, and 9) Thawing and fertilization. Each component is important, and just as links form a chain, failure of any single component can lead to failure for the entire project.

Sperm Collection

Stripping

1) Anesthetize fish with MS222 or suitable anesthetic.
2) Blot fish dry with towel to prevent water from mixing with sperm.
3) Gently massage the abdomen to expel sperm, being careful not to contaminate sperm with urine or feces.
4) Collect sperm in microhematocrit tubes, syringes, or centrifuge tubes depending on the amount of sperm to be collected.
5) Measure volume of sperm and dilute with extender solution (1:3, 1:5, 1:10, etc. depending on species, concentration, or necessity).

In some species, pressure applied under the pectoral fins can cause release of sperm. This technique minimizes contamination of sperm with urine or feces, which can cause activation of sperm or bacterial contamination. Catherization can reduce contamination of sperm, but it is often impractical. Dilution of samples in extender at the time of collection can counteract the effects of contamination by urine or water. This effect can be tested in studies of refrigerated storage.

Surgical Removal of Testis (for fishes from which sperm cannot be stripped)

1) Anesthetize fish with MS222 or suitable anesthetic.
2) Blot fish dry with towel to prevent water from mixing with sperm.
3) Surgically remove testis from fish.
4) Remove excess blood and tissue from testis.

Wayman, W. R. and T. R. Tiersch. 2011. Research Methods for Cryopreservation of Sperm. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 672-683.

5) Weigh testis.
6) Measure extender solution (10 to 20 mL per g of testis).
7) Crush or slice testis to release sperm.

To crush testis: Place the testis in a resealable plastic bag (e.g. ziplock®), remove air, and add enough extender solution to keep testis in liquid while crushing. Apply pressure to testis in a rolling motion using a small beaker, small bottle, or other round object. Strain smashed testis and extender solution through cell separator (or screen) to remove testicular tissue. Add remaining extender solution to bag to rinse. Add extender from bag to extended sperm.

To slice testis: Place the testis in a resealable plastic bag. Slice the testis into segments to release sperm, using care to avoid cutting the bag. Add half of extender solution to bag and mix. Pour contents of bag through cell separator (or screen) to remove testicular tissue. Add remaining extender to bag to rinse, strain debris and combine the extended sperm.

It is advisable to collect samples of blood plasma or serum, seminal plasma, ovarian fluid and water from the site of fish collection or spawning to measure osmotic pressure. These samples can be frozen in the field.

Motility Estimation

1) Place 2 μL of sperm on microscope slide.
2) Add 20 μL of activating solution, for example, deionized water for freshwater species (20 mOsmol/Kg) or artificial sea water for marine species (800 mOsmol/Kg)
3) Mix thoroughly.
4) Estimate percentage of progressively motile sperm using 100 or 200-× dark-field microscopy.

In some species of fish (e.g. razorback sucker *Xyrauchen texanus*), sperm remain motile for short periods of time (<10 sec). This makes estimation of motility difficult and samples may need to be evaluated several times to yield an accurate estimate. The use of activating solutions of ~140 mOsmol/Kg can increase the duration of motility in some species, but complete knowledge of the effects of osmotic activation should be understood for a species before such activation solutions are used. In other species, especially marine fishes, sperm can remain active for as long as 30 min, which simplifies estimation of motility. Ensure that sufficient dilution of sperm (e.g. > 1:10 with activating solution) is used to elicit maximal activation for each sample.

For estimation of percent motility, include only sperm that are actively swimming in a forward motion. Sperm that remain in place with only a vibratory movement should not be included. Practice the procedure to ensure that sperm movements are not due to swirling of the activating solution or Brownian movement. Some microscopic organisms (e.g. bacteria) are motile and can be mistaken for sperm by inexperienced observers (Jenkins and Tiersch 1997). Report the exact procedure used for motility estimation in sufficient detail to assist in making comparisons among studies.

Extenders and Refrigerated Storage

Extenders

Extenders have been defined as "a solution of salts, sometimes including organic compounds, which helps maintain viability of cells during refrigeration" (Graybill and Horton 1969). Extenders have been developed for many species. Hanks' balanced salt solution (Table 1) has been used successfully in our laboratory with sperm of several species including channel catfish *Ictalurus punctatus* (Tiersch et al. 1994) and simple solutions such as 1% unbuffered NaCl have been used with good results (e.g. Gwo et al. 1991).

Table 1. Ingredients of Hanks' balanced salt solution.

Ingredient	g/L
NaCl	8.00
KCl	0.40
$CaCl_2 \cdot 2H_2O$	0.16
$MgSO_4 \cdot 7H_2O$	0.20
Na_2HPO_4	0.06
KH_2PO_4	0.06
$NaHCO_3$	0.35
$C_6H_{12}O_6$ (glucose)	1.00

Extenders are formulated at an osmolality that will maintain sperm cells in a non-activated state (e.g. 280 to 300 mOsmol/Kg for freshwater species and 200 to 300 mOsmol/Kg for marine species). Report the osmotic pressure of extender solutions if possible. Before use verify that the extender solution does not activate sperm (Bates et al. 1996). With appropriate testing it is likely that particular extenders can be prepared in large batches and be stored frozen until use. Use of extenders provides increased storage time and dilutes the sperm to a greater volume, making the sperm easier to work with. Specific dilution ratios should be optimized for each species.

Extenders should be sterilized by passage through a filter or by autoclaving (if this does not affect the ingredients) and should be refrigerated. This is especially important for extenders that contain sugars. At a minimum, sterilize the water used in preparing extenders. Bacteria such as *Pseudomonas* are ubiquitous in aquatic environments and can rapidly cause deterioration of extended sperm (Jenkins and Tiersch 1997). Antibiotics can be added to extenders to reduce the growth of bacteria that reduce sperm viability (Stoss 1983). Antibiotics at high concentrations can be toxic to sperm cells, therefore, concentrations should be optimized for each species (e.g. Christensen and Tiersch 1996).

Refrigerated Storage

Sperm samples should be stored at 4 °C in an ice chest or refrigerator and care should be taken to avoid freezing the samples. In general, sperm samples should be stored in shallow containers with the lids loosely attached to allow oxygenation of the sperm cells during storage. Supplementation with pure oxygen gas has been shown to increase storage time, but could also

be inadvisable depending on the species. Containers should only be partially filled, leaving a large air space between the sperm sample and lid and should be inspected daily to provide mixing of the sperm cells. If not mixed, the cells can collect at the bottom and deteriorate.

Labeling of Straws, Goblets and Canes

Proper labeling of cryopreserved samples is essential. Usually samples are in storage for d, mo, or even yr before they are thawed. Improperly labeled samples can cause delays in processing, and even worse, could cause genetic contamination of pure stocks of fishes. The necessity for proper labeling cannot be overemphasized. The value of samples is directly proportional to the quality of labeling information and record keeping. Unlabeled or poorly labeled samples are essentially worthless.

Labeling of Straws

At the minimum, straws used for research should be labeled to indicate fish identification number, cryoprotectant, and cryoprotectant concentration. A simple method for labeling is to use straw color for identification of cryoprotectant and a system of marks on the straws to identify fish number and cryoprotectant concentration. Marking could consist of a series of dashes and dots, in which dashes represent the number five and dots represent the number one. Dashes and dots would be summed to yield the final values. For example, fish number can be identified at the top of the straw (near the factory plugging). Cryoprotectant concentration can be marked lower with sufficient space between it and the fish number to allow easy recognition (Figure 1). If possible, more sophisticated labeling, such as pre-printed straws, should be considered even for research applications. Straws intended for archiving and breeding uses should receive the best labeling possible.

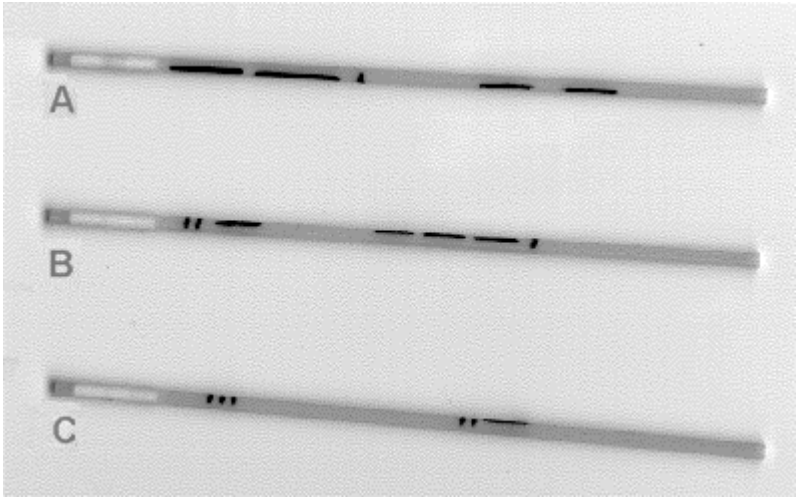

Figure 1. A labeling system for straws used for research. Straw A: fish # 11 and cryoprotectant concentration of 10%, straw B: fish # 7 and cryoprotectant concentration of 16%, straw C: fish # 3 and a cryoprotectant concentration of 7%.

Labeling of Goblets

Straws can be stored in LN_2 in plastic containers called goblets. Goblets are manufactured in numerous colors and should be labeled to identify species, date, technician, type of study and any additional pertinent information. To avoid problems in removing frozen straws, do not pack them too tightly in the goblets

Labeling of Canes

Goblets are usually attached to aluminum canes for storage in LN_2. Canes should be labeled on the top for easy identification. Labeling will decrease excessive searching of dewar contents for necessary straws. This helps to protect the straws from warming during handling.

Addition of Cryoprotectants

Background

Cryoprotectants are chemicals that allow cells to survive freezing protocols. They are grouped into two broad categories: those that are able to permeate cells and those that are not. Examples of permeating cryoprotectants include dimethyl sulfoxide (DMSO), methanol and glycerol. Examples of non-permeating cryoprotectants include sugars such as glucose or sucrose, polymers such as dextran, milk and egg proteins and antifreeze proteins such as those found in polar fishes. Use reagent-grade chemicals and report the manufacturers. Record all available information on specific lots of reagents.

Cryoprotectant Toxicity

Dependent on dose, most cryoprotectants are toxic to cells. To minimize toxic effects by cryoprotectants, they can be added slowly, at cool temperatures and should be diluted with extender solutions (e.g. 1:1) prior to addition to sperm. Some cryoprotectants can cause exothermic reactions when mixed with extenders. Care should be taken to make sure that mixtures are cool before addition to sperm. Always report cryoprotectant concentration clearly in molarity or percent (or both) and be sure to indicate if the reported concentration is before or after final dilution with extended sperm.

Equilibration Time

After the addition of cryoprotectants to sperm, time is needed for the cryoprotectant to permeate the cells. This is referred to as the equilibration time. For most circumstances it can be set at 15 to 30 min, but it can be varied depending on the type and concentration of cryoprotectant being used. If the necessary concentration of cryoprotectant is toxic to the cells, the equilibration time of rapidly permeating cryoprotectants such as DMSO can be shortened to the minimum time required for filling of straws. Always define and report equilibration time.

Equilibration Motility

To determine the approximate percentage of viable sperm after equilibration (before freezing), motility can be estimated at the start of the freezing procedure. Loss of motility can be attributed to toxic effects of the cryoprotectants. This information can help to optimize the freezing protocol by changing cryoprotectants, adjusting cryoprotectant concentration, or by adjusting equilibration time.

Filling of Straws

Background

French straws are designed to hold a specific volume (e.g. 0.5 or 0.25 mL). Manufacturers place a sealing powder surrounded by cotton plugs at one end of the straw. This area seals the straw when the sperm mixture is drawn into the powder.

Filling Individual Straws

To fill straws individually, a syringe (1 to 3-mL) can be fitted with a piece of tubing or a special adapter. This attaches to the straw and creates an air-tight seal. The straw is filled by drawing up on the syringe plunger. When the sperm mixture is ~ 0.5 cm from the bottom of the cotton plug, the straw is removed from the mixture (Figure 2). The sperm in the straw is drawn into the sealant powder between the cotton plugs. This seals the straw at the top and creates an air space within the straw. This space is necessary for safe and effective cryopreservation. The use of transparent or translucent straws greatly assists the filling process.

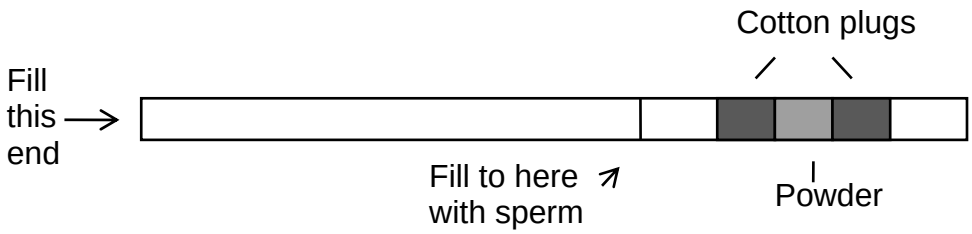

Figure 2. Diagram of standard French straw used for cryopreservation.

Filling Multiple Straws

To fill multiple straws in batches of 15 or more, a manifold of appropriate size (commercially available) is attached to a vacuum pump hose. The amount of suction is regulated by use of a valve connected to the vacuum hose by use of a “T” fitting. The straws are held together with a clamp and attached to the manifold. The flow of extended sperm can be regulated by holding the straws vertically. Filling can be completed by slowly lowering the manifold away from vertical. Once the straws are filled to within 0.5 cm from the cotton plugs they are removed from the sperm mixture. The straws are then lowered to horizontal to seal the factory-plugged end.

Sealing Straws

To seal with PVC powder: Tap the open end of the straws in PVC powder to force it into the straw. Place the straws in extender solution to gel the powder. After a short period of time, the straws can be removed from the extender and cleaned. Press the end of the straw against a flat surface to force the gel plug further into the straw (this also helps to remove excess powder from the sides) and wipe the outside of the straw.

To seal with balls: Each straw is sealed by individually forcing an appropriate-sized glass or metal ball (commercially available) into the end of the straw. The ball must be forced entirely into the straw because insufficient sealing can allow LN_2 to leak into the straw during freezing and storage. *Upon thawing, this LN_2 will vaporize and cause the straw to explode or the ball to*

be expelled from the straw at a high rate of speed. Both situations are very dangerous. As an additional precaution, always point the end sealed with a ball in a safe direction (usually down) and wear eye protection during thawing.

Handling of Straws

Straws should be handled from the factory-plugged end and should be wiped dry to keep them from sticking together when frozen. Straws sealed with balls will occupy more space in the goblets than will straws sealed with powder.

Freezing Procedures in the Field

Nitrogen-vapor Shipping Dewars

A shipping dewar is a device that allows LN_2 to be safely transported. The LN_2 is adsorbed by a filler material within the sides of the dewar (Figure 3) allowing use of cryogenic temperatures without the dangers of LN_2 spills. Nitrogen-vapor shipping dewars were designed to transport cryopreserved materials, but they can also be used to freeze samples in situations where other freezing options are not available (such as in field applications). Shipping dewars were designed to maintain cryogenic temperatures for as long as 3 wk, making these dewars useful for cryopreservation and storage in the field (e.g. Wayman et al. 1996).

Filling Nitrogen-vapor Shipping Dewars

Dispense LN_2 into the dewar until it is filled to just below the top. Allow ~30 min for the LN2 to be adsorbed in the dewar wall. Refill the dewar to the previous level. Repeat these steps until LN_2 remains in the dewar after 30 min. Pour off excess LN_2 before use. *Use caution when disposing of the excess LN_2 because it will freeze whatever it comes in contact with (e.g. human skin, floor tiles or drain systems).*

Freezing in Nitrogen-vapor Shipping Dewars

To freeze in shipping dewars, place the straws in goblets and fasten the goblets on canes. Place the canes in the canister and lower it into the dewar. Replace the shipping dewar top. After ~30 min the samples should have reached temperatures below -100 °C and can safely be moved to storage dewars. Alternatively, the samples can

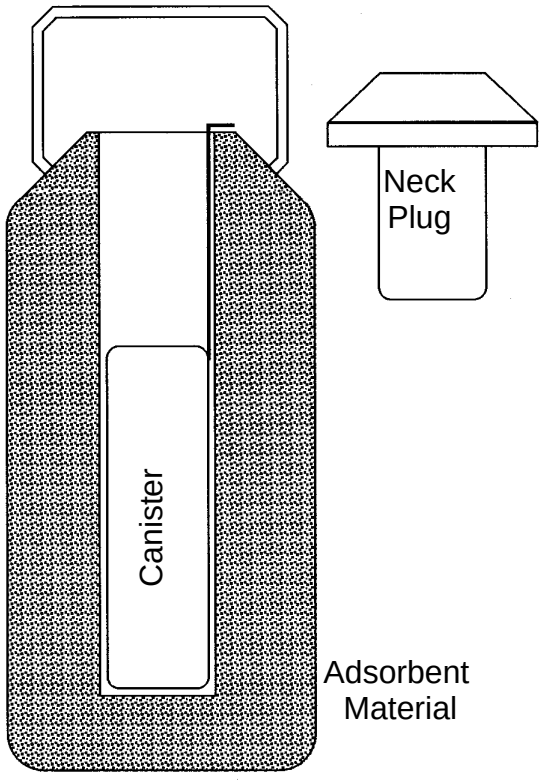

Figure 3. Diagram of a shipping dewar.

be left in the shipping dewar if a storage dewar is not available. Cooling rates can be manipulated to some extent by varying container (e.g. straw) size, position within the dewar (top or bottom) or the number of straws per goblet. In addition, straws can be frozen individually without canes or goblets. Be sure to monitor the cooling rate, for example by use of a thermocouple and recorder, and be aware that cooling rates will vary in shipping dewars due to a variety of factors including time since filling, the number of straws being frozen and the amount of use. Report the conditions used for freezing in dewars in sufficient detail for others to repeat the procedure.

Freezing Procedures in the Laboratory

Freezing in Computer-controlled Freezers

Computer-controlled freezers are manufactured by several companies (e.g. Planer, Cryomed) and cool samples by metering of cryogenic nitrogen vapor into a freezing chamber. The rate of freezing is controlled by a microprocessor. There are many different cooling protocols (some use two or more cooling rates within a program), although a simple freezing protocol with only one rate can be effective (Leung and Jamieson 1991).

1) Connect LN_2 to freezer.
2) Turn on freezer and select cooling rate.
3) Start program.
4) Place canes in freezing chamber when prompted.
5) Remove canes from freezing chamber when prompted.
6) Immediately place canes in storage dewar.
7) Allow freezer to return to start temperature.
8) Run another program or turn off freezer.
9) Remove freezer lid to allow condensation to evaporate (this is important in humid locations).

Most computer-controlled freezers allow programming of steps for insertion and removal of straws. If the freezer does not, samples should be held at the equilibration temperature or at 4 °C until the beginning of the freezing steps. If the freezer does not prompt the user to remove samples, the program should allow the samples to be held at the final temperature (e.g. -80 °C) before removal to ensure proper freezing and to allow sufficient time to transfer samples to LN_2.

Storage Procedures

General Considerations

Storage dewars are designed to store cryopreserved samples in LN_2 for extended periods of time. They use a vacuum chamber to provide insulation (Figure 4). Liquid nitrogen (LN_2) within the dewar will evaporate over time and must be replaced. The use of alarms on storage dewars is essential. The alarm sounds when the temperature at a probe raises above a certain level, indicating that LN_2 needs to be added to the dewar. Some alarms require installation and positioning of the probe in relation to the level of LN_2. This positioning should take into account the margin of safety required between the sounding of the alarm and the replenishment of LN_2. For example, would you feel safe with 1 d or 1 wk before the uppermost samples began to thaw? Be certain that the alarm remains on at all times and is tested daily. A periodic visual check of

the LN_2 level is also advised in case the alarm should fail. A log book for LN_2 additions can help identify a possible failure of the vacuum seal and assist in estimating storage costs. Ensure that the outer casing of the dewar is not punctured. The loss of vacuum will boil off the LN_2 rapidly. Rough handling can cause weakening of the inner neck area, and reduce the working lifetime of the dewar. A roller base will allow safe and easy movement of the dewar.

Removing Samples

When removing samples from storage dewars, keep the canister as far down in the dewar as possible to avoid unnecessary thawing of the remaining samples. Remove the samples quickly (proper labeling will reduce searching time) and transfer the samples to LN_2 contained in a styrofoam ice chest. Careless handling of frozen samples can allow them to warm to temperatures that allow formation of intracellular ice crystals,
which will damage the cells.

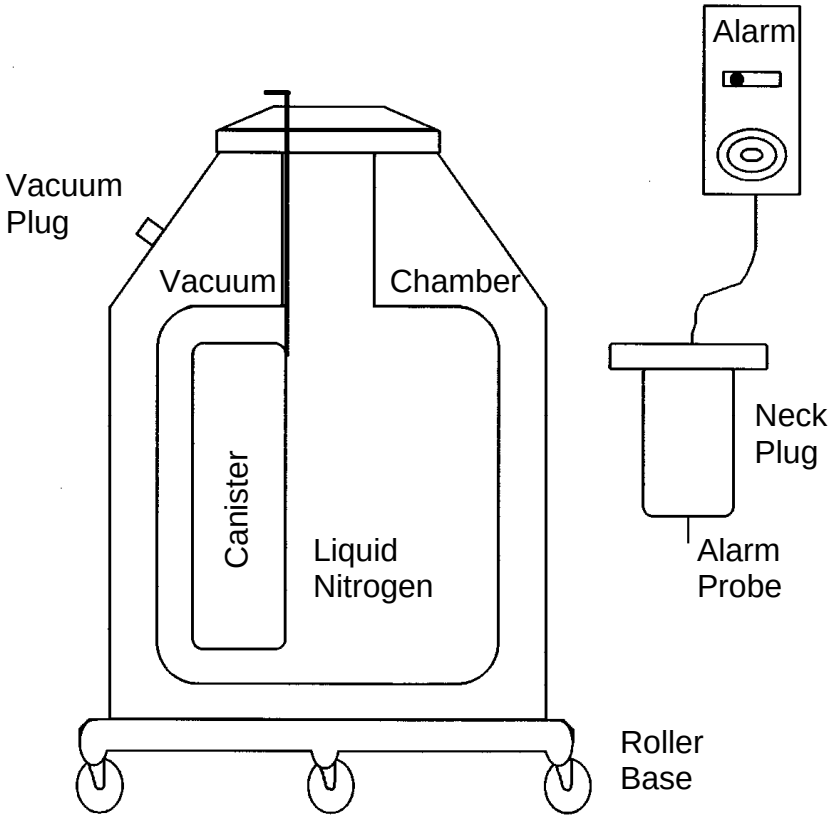

Figure 4. Diagram of a LN_2 storage dewar.

Liquid Nitrogen Safety Precautions

Always wear insulated gloves and safety glasses when handling LN_2. Never place objects cooled by LN_2 against unprotected skin. Use only containers designed for use with LN_2. Use proper transfer equipment to move and handle samples. Never use hollow rods or tubes as dipsticks, because LN_2 can be driven out of the open end.

Never cover or plug the opening of any LN_2 container. Considerable pressure will build up as the LN_2 vaporizes. Always allow proper venting. In addition, nitrogen gas can collect in closed areas, displacing the air, and creating a potential suffocation hazard. Work in well-ventilated areas.

Thawing and Fertilization

Thawing

Samples should be removed from the storage dewar and transferred immediately to a styrofoam ice chest containing LN_2. This ensures that the samples will not thaw prematurely due to handling. Hold 0.5-mL straws in a 40 to 50 °C water bath (a thermos or small ice chest will work) for ~7 sec. Specific times and temperatures should to be optimized for the particular species. We test a range of thawing temperatures (e.g. from 0 to 60 °C) to optimize protocols for each species (Wayman et al. 1998). As a rule of thumb, samples are thawed when air bubbles within the straw can move freely within the liquid. The use of transparent or translucent straws will aid in viewing the sample. If the straw is not transparent, the samples should be cool to the touch when thawed.

Sperm motility should be estimated as described above. Qualitative observations of thawed sperm can be quite helpful in evaluating protocols. For example, if the sperm cells are visibly damaged, the cryoprotectant concentration may have been too low, or the cooling rate may have been too rapid. Conversely, if the cell morphology is intact although the sperm are immotile, the concentration of the cryoprotectant may have been too high. This would be confirmed by a reduction in the equilibration motility.

Fertilization

Thawed sperm samples should be added to eggs and thoroughly mixed, and the gametes should be activated with an appropriate solution (the “dry method”). Other fertilization methods are available and should be evaluated (Urbanyi et al., this volume). Fresh sperm samples should be used to fertilize other batches of eggs to serve as a control for egg quality. After ~5 min, water should be added to water-harden the eggs. Percent fertilization should be determined to evaluate gamete quality. Estimates of sperm concentration can be made by a number of methods, and can be used to calculate the ratio of sperm to eggs, which should always be reported if possible.

Acknowledgments

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Annotated Bibliography of Developments in the Last Decade

Development of new protocols continues for aquatic species. A number of books and review articles have compiled these since the first edition of the book in 2000.

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Minimizing Microbial Contamination of Sperm Samples

Jill A. Jenkins

Methods

With the collection and translocation of gametes from aquatic species, a potential hazard exists for microbial transfer. Contamination of semen can occur during collection, processing, storage, and transport. Some preventative measures are described below for limiting the spread and amplification of microorganisms such as bacteria, viruses, fungi, mycoplasmas, and parasites.

Generally, sanitation during collection is essential. Materials and equipment used to freeze semen should be sterile. Following good practice guidelines for handling and processing samples collected for freezing is especially important for non-domestic animals where disease-free status cannot be guaranteed and unsophisticated technology is used (Russell et al. 1997).

Gamete Collection

The quality of semen may be affected by the health of the donor (AFS Fish Health Section 1997), collection technique, and the method of sample handling. Collected semen should be free from water, blood, mucus, or fecal matter. Withholding feed prior to milt collection has been suggested (Saad et al. 1988, Rana 1995).

Diluents

The ideal extender varies among fish species. Solutions should be stored at 4 to 5 °C and be used within 1 week (Rana 1995), or be frozen at -20 °C or preferably below. Optimally, the diluent should be sterilized (by filter sterilization or autoclaving). A check should be made before use that sperm are not activated by the diluent (Jenkins and Tiersch 1997).

Water Used for Making Diluents

High-purity water should be used. Concern should be given to the proper storage of laboratory water. Water can be sterilized by autoclaving (steam at high pressure and temperature). To obtain water free of bacteria and mycoplasmas, the use of 0.1-µm filters is suggested.

Cryoprotective Agents

Agents such as glycerol, dimethyl sulfoxide (DMSO), methanol and ethylene glycol are used at the appropriate final concentration (v/v) and osmolarity with the diluent. Uncontaminated agents of good chemical grade at the appropriate concentration should be used (glycerol, for example, is easily contaminated by microorganisms).

Antibiotic Solutions

If adding antibiotics to semen, add the solution to yield the final concentration per mL. Antibiotics, if in powdered form, should be dissolved in sterile distilled water. Allow for a minimum pre-established contact time before processing. There should not be an indiscriminate addition of antibiotics (shotgun approach), and those added should provide broad-spectrum

Jenkins, J. A. 2011. Minimizing Microbial Contamination of Sperm Samples In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 684-687.

activity. The selection of antibiotics is best made following cytotoxicity studies that delineate the appropriate type and concentration that favor storage of sperm from the species under study.

Packaging in Straws

Good practice guidelines for packaging and labeling of semen must be established. Leakage of contents and experimental contaminants has been demonstrated (Russell et al. 1997).

Nets, Fish-handling Equipment and Tools

When possible, air dry nets between use. Common disinfectants include chlorine (200 mg/L for 60 min or 100 mg/L for several hr), quaternary ammonium compounds such as Roccal, formaldehyde at 75-100 mg/L (3 to 4 mL of 37% formaldehyde per 40 L of water), and iodine germicides (Piper et al. 1983, Avault 1996). Clean containers are needed for milt collection, refrigerated storage, and thawing samples. Disposable plasticware can eliminate or reduce problems.

Equipment

Semen collection equipment that comes in contact with fish should be thoroughly disinfected after each use. Effective engineering controls for handling sperm samples can include general room ventilation and high-efficiency particulate air (HEPA) filters. Periodic internal and external sterilization (with 70% ethanol) of the LN_2 storage tank will reduce contamination, as infectious agents have been transferred through LN_2, and can occur from pellet-to-pellet (Thacker et al. 1984).

Water for Incubation of Fertilized Gametes and Ice for Shipping

Use disinfected or pathogen-free water supplies. Treat water from the hatchery before release as effluent.

Personnel

Workers should wear protective clothing and gloves and avoid mouth pipetting. Surfaces, hands, and tools should be washed to prevent cross-contamination. Adequately trained professionals are needed to gather reliable data such as estimates of motility, and to process specimens properly.

If gametes are to be collected at a facility that is also planning to perform analytical and diagnostic microbiology, additional rules apply. Areas should be designated for cell and bacterial cultures, media preparation, glass washing, sterilization, storage, microscopy and record-keeping. Animal facilities should be physically separated from culture facilities, and traffic should be controlled in and between areas. Separate containment areas are needed to handle hazardous and potentially contaminated cultures, and protocols must be established for disposal of contaminated samples. All media coming in contact with gamete samples, and unused samples, should be decontaminated before disposal.

Consideration must be given to the particular situation at hand. All regional, state, federal, and international guidelines for gamete transport (OIE 1997a,1997b, Code of Federal Regulations 1997) must be followed. In the United States, consult the U.S. Fish and Wildlife Service National Fish Health Policy (USFWS 1995), the basis for USFWS efforts to contain, control, and minimize impacts of fish diseases and pathogens. Systematic disinfection methods can be used at hatcheries, but this sort of control cannot be applied in natural ecosystems.

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Annotated Bibliography of Developments in the Last Decade

Since 2000 only five articles and one book chapter were found to have been published on the subject of microbial contamination in sperm. However, the broader concept of biosecurity has become prominent since 2000, especially with increased international transportation of agricultural products. A keyword search resulted in 127 articles and 5 books discussing

biosecurity since 2000, but with only 14 of those articles and 2 books addressing aquatic sciences. A selection of articles is listed below on microbial contamination of sperm and biosecurity. Clearly, additional work is necessary to incorporate the concepts of biosecurity into the field of cryopreservation in aquatic species.

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Estimating Milt Quality of Salmonid Fishes

Joseph G. Cloud

Methods

Semen evaluation is an important component of a cryopreservation program in order to cull poor quality semen samples prior to freezing and to estimate the fertility of the stored sperm after thawing. The rationale for culling poor quality semen is that the cryopreservation process is costly in terms of personnel time and materials, that space in LN_2 storage tanks is finite and that the eggs to be fertilized by the cryopreserved sperm may be quite valuable. The methods of semen evaluation to be considered are fertilization rate and sperm motility.

Fertilization Rate

The proportion of eggs that are fertilized by a given number of spermatozoa or by a standardized volume of semen provides a useful technique to measure the success of sperm cryopreservation. In many studies, the proportion of embryos that have successfully developed to the stage of retinal pigmentation (or eye-up) or have completed hatching have been used as the endpoints. These endpoints are excellent indicators of sperm quality, but for many species (like the salmonids) the time required to reach these endpoints restricts usage to the evaluation of sperm quality after thawing. For the salmonids, the proportion of eggs that has been successfully fertilized can be diagnosed after 12 to 24 hr of incubation using fixed material (1.5% glacial acidic acid in 0.9% saline solution, for long term storage fix the embryos with Stockard's solution). The blastodisc of fertilized eggs will have undergone cleavage during this time and is quite distinct. Although this procedure requires a good quality stereo-microscope and a little experience, the savings in time and incubator space is well worth the effort. Alternatively, the embryos can be fixed at about 9 d of incubation (the keel stage). At this time the formation of the embryonic axis is distinct in those embryos that are developing.

Sperm Motility

Fish spermatozoa are immotile in seminal plasma. The proportion of sperm that become motile and the degree and duration of motility following the addition of an appropriate activating solution can be correlated with fertility. Sperm motility is usually expressed as the percentage of motile sperm following activation. Alternatively, some investigators rate sperm motility on a defined scale of from 1 to 10 or with a defined classification system. In addition to motility, other characteristics such as sperm progression and the time of active motility or percent motility at varying times after activation have been used to gain additional information with which to predict the potential fertility of the sperm. As a note of caution, there are a number of examples in the literature in which sperm motility did not equate with fertility. For example, in our recent investigations in the cryopreservation of sturgeon sperm, we have observed a number of semen samples in which the post-thaw motility was greater than 50%, but were infertile in every case (Cloud, unpublished data).

Cloud, J. G. 2011. Estimating Milt Quality of Salmonid Fishes. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 688-689.

Annotated Bibliography of Developments in the Last Decade

There have been relatively few articles published since 2000 specifically addressing quality analysis of fish sperm. However, spermatozoa evaluation in mammals such as stallion, boar, and, canine is fully developed and standardized. Previous examinations and reviews in the field of aquatic species have been adopted from quality control and standardization originating from mammalian research.

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Shipping of Refrigerated Samples

Terrence R. Tiersch

It is often necessary to ship or transport refrigerated sperm or blood samples for analysis and cryopreservation. Frozen samples can then be shipped back to the original location (e.g., a hatchery) in shipping dewars for use at a later date. Typically it is essential that the samples remain unfrozen prior to cryopreservation. Although this would seem to be simple, our experience indicates that samples are often destroyed by improper practices during shipment.

There are several precautions that can be used to prevent such losses. First, ensure that the samples do not come in direct contact with the ice or gel packs in the cooler. A simple cardboard divider can be useful for this (Figure 1), as is the precaution of wrapping the gel packs in paper towels. It is important to keep in mind that, at least for warmwater fishes, we are trying to prevent the samples from becoming heated, not in keeping them as near freezing as possible. For valuable samples, a temperature data logger or a thermometer that records maximum and minimum temperatures could be included.

Collection and shipping of samples often requires more time than expected. It is a good idea to verify the address and phone number of the recipient well before the d of shipping. If storage time is a constraint, collections can be scheduled to finish at the time of shipping, although this requires careful planning. We have found that the best approach is to collect and ship samples in a preliminary rehearsal to identify problems and to avoid loss of valuable samples or data. This rehearsal should be performed using the same procedures and schedule planned for the actual samples, especially if factors such as automobile traffic can interfere with the schedule.

The recipient should be notified when the samples are sent and should know when and where the samples will be delivered. Samples should be evaluated before they are shipped, and should be evaluated again immediately upon receipt. With sperm, for example, percent motility and general charateristics (e.g., color, presence of gelling) can be evaluated.

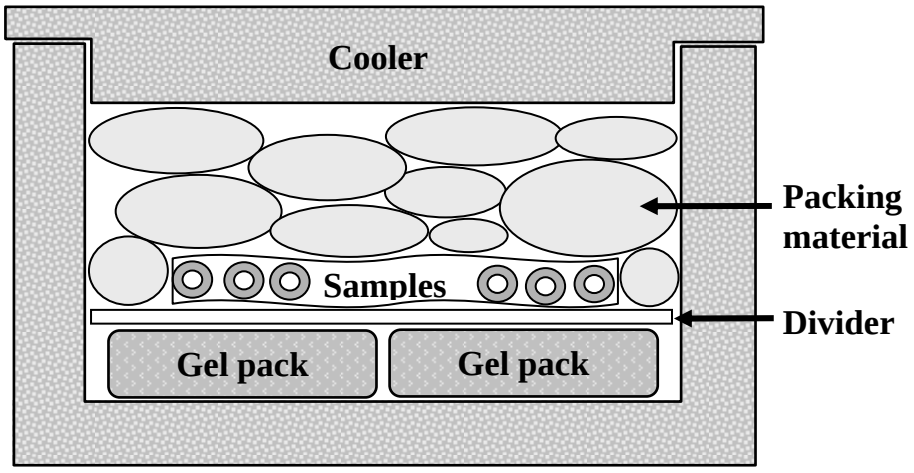

Figure 1. Proper packaging of samples for refrigerated storage during shipment. Samples should be placed in a waterproof bag with protection from freezing during shipment.

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Annotated Bibliography of Developments in the Last Decade

Shipping of refrigerated sperm or blood samples for analysis or cryopreservation is a standard practice. Since the original publication of this chapter in 2000, continued research in this field has been completed for the shipment of a number of biological samples. More recently, the use of radio frequency identification systems has provided greater efficiency in shipping of time-sensitive biological samples.

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New Chapter

Simple Extenders for Refrigerated Storage and Cryopreservation of Channel Catfish Sperm

William R. Wayman, Chester R. Figiel Jr. and Terrence R. Tiersch

Introduction

Genetic improvement of channel catfish *Ictalurus punctatus* broodstock is needed to maintain desirable characteristics for aquaculture (e.g., growth rate, feed conversion), to avoid problems associated with inbreeding (e.g., reduction of immune system function), and to ensure conservation of genetic resources. Additionally, hatchery techniques are required to increase the cost effectiveness of aquaculture breeding programs. For example, buffered extender solutions have been the used for refrigerated storage and cryopreservation of channel catfish sperm (Guest et al. 1976, Tiersch et al. 1994, Christensen and Tiersch. 1996). These methods can be used for selective breeding to improve commercially important traits. However, the typical farm does not have the resources necessary for the preparation of extenders used for refrigerated storage and cryopreservation of sperm. Simplification of processes, such as the use of minimal extenders, could advance development of breeding programs and improve production economics. To that end, this study evaluated simplified sperm extenders for use in the refrigerated storage and cryopreservation of channel catfish sperm. Our goal was to identify extenders based on readily available food-grade ingredients useful for artificial spawning that would be accessible to a wide range of farmers and breeders. The objectives were to evaluate the effects of extender composition on refrigerated storage of sperm, cryopreservation of sperm, fertilization percentage, and growth of fingerlings.

Experimental Approach

Extender Preparation

Three extenders were examined (Table 1) made from materials found normally at local grocery stores, and compared these with a widely accepted extender, Hanks' balanced salt solution (HBSS) prepared with eight reagent-grade chemicals (Sigma Chemical Corp., St. Louis, Missouri, USA) (Tiersch et al. 1994). All extenders were adjusted to an osmolality of 290 mOsmol/Kg, although no attempt was made to adjust pH.

Table 1. Composition of extenders used with channel catfish sperm.

Solution (g/L)	$NaHCO_3$	NaCl	pH
Arm & Hammer baking soda®	13.82	--	8.5
Morton salt®	--	9.17	6.0
Combined (baking soda & salt)	6.91	4.59	8.5
Hanks' balanced salt solution	(recipe in Tiersch et al. 1994)		6.5

Sperm Collection

Testes were removed from six mature channel catfish during the normal spawning season in Southern Louisiana (May, 1996). Each testis was cleaned, weighed, and crushed in an

Wayman, W., C. R. Figiel Jr. and T. R. Tiersch. 2011. Simple Extenders for Refrigerated Storage and Cryopreservation of Channel Catfish Sperm. In: *Cryopreservation in Aquatic Species, 2nd Edition*. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 692-697.

extender at a ratio of 1 g of testis to 20 mL of extender. The extender/sperm mixtures were filtered through a sieve (Cellector®, E-C Apparatus Corp Milford, MA) to remove pieces of testicular tissue. Samples were stored in 100-mL plastic beakers at 4 °C until use in experiments.

Motility Estimation

Motility was estimated using 200-x dark-field microscopy immediately after the activation of 2-µL of extended sperm with 20-µL of deionized water. Percent motility was defined as the percentage of progressively motile sperm observed during 30 sec within a sample. Sperm that vibrated in place were not considered to be motile.

Refrigerated Storage

To determine the effect of extender composition on the retention of motility over time, 30-mL sperm samples from six channel catfish were stored in 100-mL plastic beakers at 4 °C. The samples were gently swirled and motility estimated daily until it was no longer present.

Cryopreservation Methods

Sperm from five channel catfish were frozen in 0.5-mL straws (IMV International Corp. Paris, France) on aluminum canes with five straws per goblet. Methanol was used as the cryoprotectant at a concentration of 5% (equilibration time, 45 min). Sperm were held in extender for less than 6 h before cryopreservation. We cryopreserved the samples using a computer-controlled freezer (Kryo 10/16, Planer Products Ltd., England) at a rate of –40 °C/min. After reaching –80 °C/min samples were transferred into liquid nitrogen for storage (Christensen and Tiersch 2005). Samples were stored for 24 hr before use in fertilization trials.

Fertilization and Growth Trials

Female channel catfish were induced to spawn by intraperitoneal injection of synthetic luteinizing hormone-releasing hormone (LH-RHa, Sigma Chemical Corp.) at a dose of 100 µg/ Kg body weight. Eggs were collected into greased bowls containing HBSS (Bates et al. 1996). Between 10 and 20 mL of eggs were aliquotted per sample. Samples were thawed in a water bath at 40 °C for 7 sec. Fresh (0.45 mL) or cryopreserved (0.50 mL) sperm were added to eggs and mixed. Water was added to initiate fertilization and water hardening. Fertilization percentage was determined by dissolving the egg matrix with sodium sulfite (15 g/L) for 40-60 sec, placing the eggs on a light box, and counting the number of neurulated embryos at 24-27 hr after fertilization. After hatching, fry were held at equal densities in replicate tanks in an indoor recirculating system for 3.5 months before measurement of length (total and standard) and weight.

Statistical Analysis

All percent motility values were arcsine-square root transformed prior to analysis. Motility data from the refrigerated storage study was compared using a repeated-measures analysis of variance (NCSS 2000, NCSS, Kaysville, Utah, USA). In the cryopreservation study, differences were examined in initial, equilibration, and post-thaw motility using a one-way ANOVA (NCSS 2000) for each analysis. Differences among the treatments in the fresh and cryopreserved sperm fertilization trials were examined with a one-way ANOVA. A randomized block design was used to examine differences in weight, standard length, and total length in the

growth study to address variation in rearing conditions. Means were separated using a Tukey-Kramer multiple comparison test, and were considered significant at the $P < 0.05$.

Results

Refrigerated Storage

During the study, sperm stored in HBSS retained significantly ($P < 0.0001$) higher motility than did sperm stored in other extenders (Figure 1). There were no differences in initial motility ($P = 0.1146$) or equilibration motility ($P = 0.2630$) among the extenders. Sperm cryopreserved in HBSS or the combined extender had significantly higher ($P < 0.0001$) motility than did sperm in baking soda or salt alone.

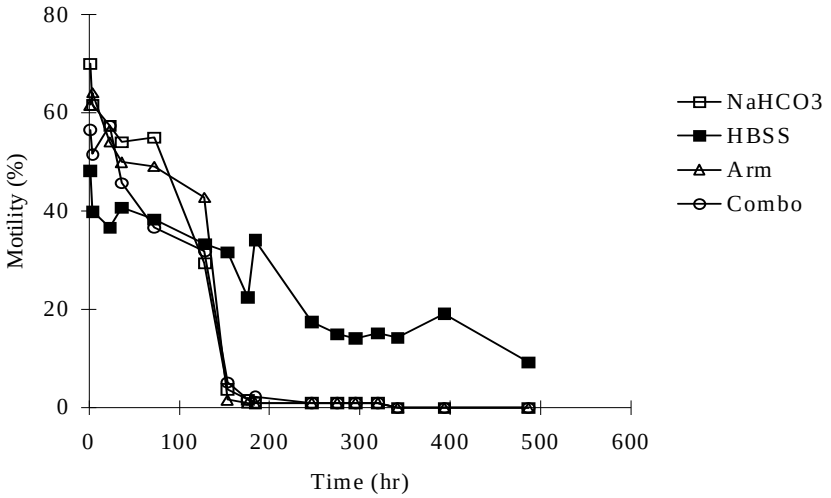

Figure 1. Percent motility of channel catfish sperm stored at 4 °C in four extender solutions. Each point represents the mean of six fish. Sperm stored in HBSS (closed squares) retained motility significantly longer ($P = 0.0001$) than in the other extenders.

Cryopreservation

There were no differences in initial ($P = 0.1146$) and equilibration ($P = 0.2630$) motility among the extenders tested. Sperm cryopreserved in HBSS and the combined extender had higher ($P < 0.0001$) post-thaw motility than did sperm in baking soda or salt (Table 2).

Table 2. Mean (± SD) motility values for sperm frozen in 5% methanol and four extenders. Equilibration was 45 min. Sperm were thawed at 40 °C for 7 sec.

	Motility (%)		
Solution	Initial	Equilibration	Thawed*
Arm & Hammer baking soda®	70 + 6	52 ± 21	3 ± 2^{B}
Morton salt®	53 + 18	63 ± 8	2 ± 2^{B}
Combined (baking soda & salt)	58 + 19	45 ± 11	9 ± 4^{A}
Hanks' balanced salt solution	44 + 17	51 ± 11	13 ± 3^{A}

* Motility estimates sharing letters were not significantly different ($P > 0.05$).

Fertilization and Growth Trials

There were no differences in the fertilization rates among extenders for fresh (P = 0.8002) or cryopreserved (P = 0.3341) sperm (Table 3).

Table 3. Mean (± SD) percent fertilization of refrigerated and cryopreserved sperm. There were no differences in fertilization among extenders for refrigerated (P = 0.8002) or cryopreserved (P = 0.3341) sperm.

	Percent Fertilization	
Treatment	Refrigerated	Cryopreserved
Arm & Hammer baking soda®	80 ± 17	81 ± 12
Morton salt®	82 ± 15	84 ± 3
Combined (baking soda & salt)	83 ± 11	68 ± 26
Hanks' balanced salt solution	84 ± 10	86 ± 13

There were no significant differences in weight (P = 0.9131), standard length (P = 0.7882), or total length (P = 0.8082) of 3.5-month-old juveniles produced from refrigerated sperm (Table 4).

Table 4. Mean (± SD) weight and length of fish produced from refrigerated sperm. There were no differences among the treatments (weight, P = 0.9131; standard length, P = 0.7882; total length, P = 0.8082)

		Length (mm)	
Treatment	Weight (gm)	Standard	Total
Arm & Hammer baking soda®	0.91 ± 0.38	39.7 ± 6.3	48.8 ± 7.2
Morton salt®	0.91 ± 0.31	39.3 ± 4.8	48.9 ± 5.6
Combined (baking soda & salt)	0.92 ± 0.28	39.6 ± 4.4	49.1 ± 5.5
Hanks' balanced salt solution	1.08 ± 0.26	42.4 ± 3.9	52.4 ± 4.6

Potential for Use of Simple Food-Grade Extender Ingredients

This study illustrates that simplified extenders can be used for the refrigerated storage and cryopreservation of channel catfish sperm. Sperm extended in commercially available food-grade preparations of sodium bicarbonate and sodium chloride, or a combination of the two had similar motility and fertilization rates. These rates were similar to those of sperm extended in HBSS, an extender that requires eight ingredients (Tiersch et al. 1994), and has been used extensively for refrigerated storage and cryopreservation of sperm of channel catfish and other species (Guest et al. 1976, Tiersch et al. 1994, Tiersch et al. 1996, Christensen and Tiersch 2005). Previous work has demonstrated the need to maintain the solution osmolality above 290 mOsmol/Kg to preserve sperm quality during storage (e.g., Bates et al. 1996). Additionally, growth and survival of fry produced from cryopreserved sperm were not affected by the extender indicating that sodium bicarbonate and sodium chloride can produce viable offspring. The important distinction is that these simple extenders did not maintain sperm quality as long as HBSS did during refrigerated storage. Based on these results, the use of salt or baking soda as extenders for refrigerated storage prior to freezing should be limited to 3-4 d, and should be evaluated before such use. The HBSS may offer a longer refrigeration period before freezing.

Storage of sperm using these simple extenders offers some advantages. Salt and baking soda are cheaper (considerably so, if cost estimates are made using the calculations provided by Caffey and Tiersch 2000) and are more readily available than the reagent-grade ingredients used to prepare HBSS. In addition, these extenders offer utility when in the field or in conditions with economic or other constraints (such as when working on a tight budget or at non-research hatcheries). Because food-grade salt and baking soda from large global companies were used, reliable quality of such ingredients should be available around the world. In addition, such extenders would likely be useful for a variety of species (e.g., Mongkonpunya et al. 1996, Kwangtong and Bart 2006, Adeyemo et al. 2007), given that preliminary testing of the type done in this study is performed prior to commitment of valuable resources.

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Extender Solutions for Sperm of Salmonid Fishes

Joseph G. Cloud

Considerations for Salmonid Fishes

In order to cryopreserve sperm, the semen is normally diluted (1:1 or 1:3) with a freezing solution. The basic component of this solution is a semen extender or diluent that has the approximate osmotic properties of seminal plasma and a cryoprotective agent. Additionally, the freezing solution may contain a buffer and other additives that increase post-thaw fertility.

Semen Extenders

A semen extender is a physiological solution that does not induce sperm activation. The semen extenders used in freezing solutions have ranged in complexity from a solution that mimics the composition of seminal plasma to a simple glucose solution. Investigations using freshwater or marine species have demonstrated that the composition of this solution affects post-thaw fertility of the sperm, but there does not appear to be a single extender that maximizes fertility for all species. The one point that is clear in the literature is that the more complicated solutions are not necessarily the best.

Cryoprotectant Agents

The freezing solutions must also contain a cryoprotective agent. Of the cryoprotective agents that have been tested for fish sperm, one of three compounds, (glycerol, dimethyl sulfoxide (DMSO) or methanol) have usually proved to be most effective. Each of these compounds is relatively small and enters cells quickly; therefore little or no equilibration time is required. Additionally, although the presence of these small molecular weight compounds are required for survival during the freezing and thawing phases of cryopreservation, the compounds are potentially toxic. They should be added to the seminal plasma at the optimal temperature and at the final concentration to afford the greatest protection during freezing and thawing without significant reduction in fertility. To date, the specific type and concentration of cryoprotectant agent that provides the maximum protection and is least toxic is ultimately identified by fertility testing extended semen before and after freezing.

Buffer

In the cryopreservation of mammalian spermatozoa, the type and amount of buffer in the freezing solution is an important parameter in the maintenance of fertility. In sperm of rainbow trout *Oncorhynchus mykiss*, the influence of pH in the freezing medium appears to be a source of variation in the resultant fertility post-thaw. The pH may not be an important factor unless the post-thaw fertility is low and research has found that freezing medium without buffer was as good or superior to media with buffer.

Membrane Stabilizers

A number of compounds have been added to the freezing solution. These components include bovine serum albumin and promine D. For a number of salmonid species, the addition of

Cloud, J. G. 2011. Extender Solutions for Sperm of Salmonid Fishes In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 698-700.

avian egg yolk to the freezing medium results in a significant improvement in post-thaw fertility. Sperm frozen in media containing 5, 10 or 20% egg yolk had no significant difference between post-thaw motility, although sperm frozen in the 10% egg yolk had the highest fertility of the three media. The conclusion is that the addition of egg yolk has a sparing effect on the spermatozoa. However, this observation for rainbow trout sperm has not been consistent for spermatozoa of other species.

Recipe for a Simple Glucose Freezing Solution

This isotonic freezing solution is composed of 300 mM glucose, 7.5% DMSO and 10% egg yolk. The solution should be made far enough in advance to allow it to equilibrate to 4 °C prior to use. The total volume of solution needed will be dependent upon the number of samples to be frozen. Because three parts of freezing solution are normally added to one part of semen, the minimal amount of freezing solution should be three times the volume of the semen to be frozen.

To make 100 mL:

Completely dissolve 5.4 glucose (dextrose) in distilled water and bring to a total volume of 100 mL. Separate the egg white from the yolk and place the resultant yolk sac on a large piece of filter paper. Carefully rupture the membrane and allow the yolk to flow into a beaker (or some other container). To 50 mL of glucose solution, add 10 mL of DMSO and 13.3 mL of egg yolk. Add additional glucose solution to bring the final volume to 100 mL and carefully mix. Centrifuge the mixture briefly to remove sediment and keep the solution refrigerated.

Annotated Bibliography of Developments in the Last Decade

Several different extender solutions have been used in investigations since publication of this work in 2000. A majority of these studies compare and adjust the functional composition of the extender solution. Although the significance of extenders has been widely accepted, to our knowledge, there is no current review on their improvements within the past decade.

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Packaging and Identification of Sperm Samples

Joseph G. Cloud

Animal scientists use polyethylene straws with a capacity of 0.25 mL (bovine mini straw) or 0.5 mL (bovine medium straw) to preserve the semen of a number of different species of domestic livestock. With this container the extended semen is drawn up into the straw and the ends are sealed. Because of the geometrical structure of the straw, the extended semen will equilibrate with the temperature of its environment very quickly. Therefore the semen in straws will freeze and thaw readily. Because of their usage in animal agriculture, the semen straw is readily available and can be easily identified and stored. We have found that a straw printer is helpful to provide a complete description of the origin of the semen in each straw. We routinely include the identification of the source of the male (if you want preprinted straws, MVE will print straws and send them overnight). The straws are printed prior to filling and are placed in the refrigerator at the same temperature as the semen and freezing solutions.

For a small number of straws, individual straws can be filled using a syringe fitted with a piece of tubing. The tubing is attached to the end of the straw and the semen plus freezing solution is drawn into the syringe leaving an air space at the end for expansion during the freezing process. The open end of the straw is closed using sealing powder.

For larger batches of straws, a manual filling system developed for the bovine industry is used. Ideally, for coldwater fishes such as Salmonids, the filling procedure should be conducted in a cold room at 4 °C. However, this condition is not always available. Therefore as a minimum, all materials and containers should be stored in the refrigerator at 4 °C until used, and the filling procedures conducted on a tray of ice.

Materials

Large plastic tray (like a 61 cm x 61 cm autoclave tray) filled with ice (4 °C)
Drying tray (22.9 cm x 33 cm x 5.1 cm, but a glass cake pan will also suffice)
Test tubes in a rack within the ice (above)
0.5-mL straws
Vacuum pump
Filling nozzle (15 pins for medium straws)
Straw clips (1 for every 15 straws)
Bubblers with troughs (1 bubbler and trough for each sample)
Bubbler stand
Sealant

1) Place the drying tray on ice (the large plastic tray) and allow it to equilibrate to 3 °C to 4 °C. Check with a thermometer.
2) Connect the 15-straw filling nozzle to the vacuum pump.
3) Into each clip insert 15 straws with the pre-sealed ends oriented in the same direction.
4) Assemble the bubbler and trough in the bubbler stand. Place the stand on the drying tray (this glass tray acts as a chilled platform on which to work).

Cloud, J. G. 2011. Packaging and Identification of Sperm Samples. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 701-702.

5) Place the test tube rack with tubes and the container of freezing solution in the ice and allow them to equilibrate.
6) Transfer the semen from one male to a tube. For every 1 mL of semen, add 3 mL of freezing solution (the freezing solution should be added slowly, and the tube should be agitated to facilitate mixing).
7) Thoroughly mix the semen and the freezing solution.
8) Pour the semen solution mixture into the bubbler trough.
9) Place the end of the straws into the bubbler trough. Attach the pre-sealed ends of 15 straws in a clip to the filling nozzle and draw the semen solution mixture into the straws.
10) When the straws fill to the pre-sealed end, release the suction hole and remove the straws from the nozzle. Do not invert.
11) Place the straws onto the combs of the bubbler.
12) Remove the straws and fill the open end of the straws with sealant powder.
13) Remove the straws from the clip and wipe off any sealant from the straws.

Annotated Bibliography of Developments in the Last Decade

Since the initial publication of this work in 2000, numerous packaging studies have been published in the field of food science research, however, studies of packaging and quality control of cryopreserved products are lacking. The use of barcodes for identification in human medicine and process control engineering has been well developed, which could serve as an example for sperm sample identification. Automatic loading-coding systems from Cryo Bio System (Paris, France) and Minitube of America (Verona, Wisconsin) with high efficiency and bio-security deserve more attention in the future.

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Cryopreservation Methods for Sperm of African Catfish Formerly Known as Sharptooth Catfish

Béla Urbányi, András Dinnyés and István Magyary

Methods

All experiments were carried out in the laboratory of Fish Physiology, Institute for Animal Husbandry, University of Agricultural Sciences, Gödöllö, Hungary in 1993 to 1996. Experiments for the development of a more efficient freezing method are described in detail (Urbányi et al.) within this book. Technical details of the most efficient methods are presented here.

Collecting the Sperm

The fish were injected with two balls (25 to 30 mg) of artificial gonadotropin releasing hormone (GnRH)-analogue [D-Ala^6,Pro^{9}NEt](Interfish Ltd, Hungary) and were incubated for 12 hr. After incubation, the fish were killed and the testes were removed. If only a small amount of sperm was needed a part of the testis was removed from anaesthetised live animals. The testis was cut into small pieces and pressed through a fabric net into a sterile dry Petri dish. The sperm was frozen as soon as possible, although it could be stored for a few hr in the refrigerator (0 °C to 4 °C).

Preparation of the Diluent

The extender, also used as a cryoprotectant, included 6% fructose (6 g of fructose in 100 mL of distilled water) and 10% dimethyl sulfoxide (DMSO) or 10% dimethyl acetamide (DMA) (expressed as final concentrations when mixed with sperm). The pH of the diluent (7.73) was established with $NaHCO_3$ buffer. It was important to mix the extender and the cryoprotectant before adding the buffer to ensure proper pH.

Equilibration

The sperm:diluent ratio was 1:1. The sperm were added to the diluent in a sterile vessel and mixed gently. Equilibration time was 10 min and took place at 3 °C. After equilibration, the sperm were drawn into 0.25-mL plastic straws, without creating air bubbles within the straw. Only 200 μL of diluted sperm was drawn into straws so the end remained empty to avoid contact with water during thawing.

Freezing

The straws were placed onto a copper tray that was placed into a programmable freezer. The following freezing rate was used: cooling at -4 °C per min from 3 °C to -4 °C, followed by cooling at -11 °C per min from -4 °C to -80 °C (Steyn and Van Vuren 1987, Magyary et al. 1996). At the end of the program there was a holding period at -80 °C during which the tray was removed from the freezer and the straws were dropped into LN_2 for storage.

Urbányi, B., A. Dinnyés and I. Magyary. 2011. Cryopreservation Methods for Sperm of African Catfish Formerly Known as Sharptooth Catfish . In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 703-705

Thawing

Sperm were thawed in a 40 °C waterbath for 5 sec. After fertilization, motility of the sperm was observed using a microscope and 50% motility was obtained using DMSO and 20 to 30 % motility was obtained using DMA (Urbányi et al. 1999).

Fertilization

One mL of eggs was fertilized with 1 straw of thawed sperm. The difference in the motility percentage between sperm suspended in the two cryoprotectants did not cause significant differences in fertilization, so both were used. In the case of DMSO, wet fertilization was recommended instead of dry. This means that water was poured onto the eggs immediately before the sperm were added, resulting in 90 to 95% fertilization. The fresh sperm (control) yielded 100% fertilization.

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Annotated Bibliography of Developments in the Last Decade

Clariid catfishes have emerged as one of the most important groups of farmed catfish in the world. Since the publication of this book in 2000 there have been relatively few papers published on the cryopreservation of sperm of African catfish. A new chapter in this revised edition has been added to further address sperm cryopreservation in this species.

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Cryopreservation Methods for Sperm of the Common Carp

István Magyary, András Dinnyés and Béla Urbányi

Methods

Collecting and Handling Sperm

All experiments were carried out in a commercial hatchery at Dinnyés, Hungary in June 1994, 1995 and 1996. Experiments for the development of a more efficient freezing method are described in Magyary et al. (this volume). Technical details of the most efficient method are presented here. Spermiation was stimulated by injection of 2 mg of carp pituitary extract (in 0.65 % NaCl) per Kg body weight 24 hr prior to stripping at 20 to 22 °C water temperature. Milt samples were transported and stored separately in 2 to 5-mL doses in plastic Petri dishes (90 mm) in a styrofoam box on ice. Sperm samples showing reduced motility upon transportation and storage were exposed to oxygen in a sealable plastic sack (3 L) for 30 min at 0 °C.

Evaluation of Sperm Quality

Motility of intact sperm was evaluated (the percentage of spermatozoa showing progressive movement within 10 sec of addition of water) using a light microscope at 100-X magnification prior to freezing. Only samples showing high motility (> 80 %) were used for freezing.

Dilution

The most efficient diluent was a modified version of Kurokura's Extender 2 (360 mg NaCl, 1000 mg KCl, 22 mg $CaCl_2$, 8 mg $MgCl_2$, 20 mg $NaHCO_3$ dissolved in 100 mL of distilled water at pH 8.0) (Magyary et al. 1996) and DMSO in 10 % (v/v) final concentration. The dilution rate was 1:9 for sperm:diluent. The sperm and the diluent were kept at 0 °C prior to dilution.

Equilibration

The diluted sperm was equilibrated for 10 min at 0 °C. The motility of equilibrated sperm was evaluated. Only samples with motility of >50 % were used for freezing. During equilibration diluted sperm were drawn into 0.5-mL plastic straws. The end of the straw (0.5 cm) was not filled to avoid contact of sperm with water during thawing.

Freezing

Diluted and equilibrated sperm were frozen in plastic straws in a programmable freezer (Cryocell 15, BLS, Hungary) using the following program: cooling at 4 °C per min from 0 °C to -4 °C, followed by cooling at -11 °C per min from -4 °C to -80 °C (Steyn and Van Vuren 1987). After reaching -80 °C, samples were plunged directly into LN_2 (-196 °C) for further storage.

Magyary, I., A. Dinnyés and B. Urbányi. 2011. Cryopreservation Methods for Sperm of the Common Carp. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 706-708.

Thawing

Frozen straws were thawed in a 40 °C waterbath for 10 sec. Motility and fertility of thawed sperm were tested. Fertilization was carried out immediately after thawing.

Fertilization

Eggs were fertilized in 20-mL plastic Petri dishes (1.5 to 2.0 x 10^3 eggs in each) or in 2-L plastic vessels (1.5 to 2.0 x 10^5 eggs in each). Pond water was added to the eggs, followed within 1 to 2 sec with the thawed sperm. The optimal ratio of thawed sperm:eggs:water was 1:20:20 in volume (1 mL of thawed sperm contained 0.1 mL of milt). After 1 min of gentle mixing, the water was replaced by Woynarovich solution (0.3 % urea and 0.4 % NaCl in water) to avoid stickiness of the fertilized eggs.

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Annotated Bibliography of Developments in the Last Decade

Common carp represent a large proportion of cultured fishes around the world. Since the publication of this book in 2000 there has been considerable expansion of technologies and research in gamete preservation for this species.

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Cryopreservation of Sperm of the Mekong Giant Catfish

Krit Mongkonpunya, Thusanee Pupipat and Terrence R. Tiersch

The Mekong giant catfish *Pangasius gigas* is considered to be an endangered species in Thailand. Because of the short duration of harvesting (spawning) season (in May) and declining harvests, gametes are difficult to obtain, especially eggs. Thus, eggs and fresh (control) sperm used in fertilization tests were often provided by *Pangasius hypophthalmus* (Mongkonpunya et al. 1992) or *Clarias macrocephalus* (Mongkonpunya et al. 1995). To obtain sperm for cryopreservation, abdominal stripping was applied ~10 hr after a sec injection of domperidone and suprefact (Pholprasith 1996). In cases where the ripe male (*P. gigas*) was slaughtered by fisherman, testicular samples were also used (Mongkonpunya et al. 1995).

The stripped semen was collected into oven-dried beakers (250-mL) or 250-mL screw-cap bottles (Duran, Schott, Germany). Samples were diluted immediately with an equal volume of 0.9% NaCl or modified Hanks' balanced salt solution (HBSS), prepared at ~ 300 mOsmol/Kg (Mongkonpunya et al. 1995). The diluted samples were stored refrigerated (4 to 6 °C) in loosely capped bottles. Sperm volume was less that 100 mL per bottle, and the samples were agitated frequently to ensure adequate aeration during storage. The testicular sperm were obtained by cutting the ripe testicular lobules into small pieces and placing them in a plastic bag with an equivalent (w:v) volume of extender, crushing to yield a homogenous sample and filtering through nylon netting of 0.5-mm mesh. Thereafter, the testicular sperm were treated as described for stripped sperm. Sperm preparation, collection and dilution were completed within 5 min for stripped samples and 15 min for testicular sperm. To minimize bacterial and fungal contamination of diluted samples during handling prior to cryopreservation, the antibiotics streptomycin (5 mg/mL) and penicillin (800 IU/mL) were added to the extender.

For cryopreservation, the sperm were diluted with the extender and sufficient dimethyl sulfoxide (DMSO) to produce a desired final concentration. The final dilution ratios (sperm:extender) were 1:3 (v:v) for stripped sperm and 1:10 (g:mL) for testicular sperm. The diluted samples had cell densities of 4.2×10^9 sperm per mL and were mixed thoroughly and placed into cryotubes of 2-mL or 5-mL capacity. Each tube was filled to about 90% of capacity. The equilibration time (between the addition of DMSO and the initiation of freezing) was set at 15 min to maximize the penetration of DMSO while minimizing toxic effects on sperm. Following equilibration, the cryotubes were placed on aluminum canes in a canister suspended within the neck of a liquid nitrogen (LN_2) storage tank (35 HC, Taylor Wharton, Indianapolis, Indiana). We ensured that the lower tubes were not in contact with the surface of the LN_2; all tubes were in nitrogen vapor only. The average freezing rates were measured with a type-T thermocouple (positioned at the middle of a tube) and a digital thermometer (Atkins Technical Inc., Gainesville, Florida). After the temperature in the upper tube (the slow freezing rate) reached -80 °C, the canister was plunged into liquid nitrogen and stored until utilization in fertilization tests.

If females of *P. gigas* were not available for fertilization experiments, female of *P. hypophthalmus* or *C. macrocephalus* were injected in the dorsal musculature with Domperidone (10 mg/Kg) and Suprefact (10 μg/Kg). We collected eggs 10 to 12 hr after injection by applying gentle pressure along the abdomen. We fertilized the eggs within 30 min of collection and used plastic bowls for fertilization and incubation of eggs.

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Annotated Bibliography of Developments in the Last Decade

The Mekong giant catfish *Pangasius gigas* is presently considered to be critically endangered, and the updated references for this chapter include studies which have focused on genetic diversity, DNA markers, and acoustic telemetry tracking of this fish, but not on sperm cryopreservation. For publicly available information on the status of the Mekong giant catfish, including the 2006 agreement to cease fishing for it in Thailand, use the search function of the Mekong River Commission website, *www.mrcmekong.org*.

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Cryopreservation Methods for Embryos and Larvae of the Pacific Oyster

Jin-Chywan Gwo

Gametes were extracted from individual mature oysters after careful removal of the upper oyster shell. Gametes were removed from the gonad using a clean pipette. The sex and sexual maturity of individual oysters was determined by observing a smear of gonad material under a light microscope. Gametes were combined from 10 or more oysters of the same sex. The eggs were fertilized with an optimized sperm concentration. The fertilized eggs were washed and filtered to discard supernatant seawater and excess sperm.

Gastrulating embryos or trochophore larvae were suspended in 10% dimethyl sulfoxide or 10% propylene glycol in 25-ppt filtered seawater for 10 min at room temperature. The suspension was adjusted to obtain a final density of 1500 individuals per mL. The suspension was aspirated into 0.5-mL plastic straws and cooled from 15 °C at a cooling rate of -1.5 to -2.5 °C per min to transition temperatures (-30 to -40 °C). Seeding was induced at -7 °C. The straws were plunged directly into liquid nitrogen for long-term storage.

The straws were immersed in a gently stirred waterbath at room temperature. Filtered seawater was added slowly to dilute the thawed embryos and they were incubated at 20 °C for 2 to 3 d. The presence of active-swimming veligers was regarded as an indicator of a successful freezing protocol.

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Annotated Bibliography of Developments in the Last Decade

Since publication of this book in 2000, more studies have been reported that address cryopreservation of eggs, embryos, and larvae in oysters. Species such as the Pacific oyster *Crassostrea gigas*, eastern oyster *Crassostrea virginica*, and pearl oysters *Pinctada fucata* are important economic species distributed around the world. Therefore, gamete cryopreservation can be a useful technique to enhance the oyster industry worldwide.

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Cryopreservation Methods for Embryos of the Pacific Oyster

Ta-Te Lin and Nai-Hsien Chao

Oyster sperm and eggs were collected from male and female oysters and cultured in seawater of 34 ± 2% salinity. Embryos were obtained from the collected eggs after artificial fertilization and were cultured in seawater at 28 °C for 4 hr. During fertilization, eggs were examined under a microscope, and the suggested number of sperm surrounding the egg to assure ideal fertilization was ~ 5 ± 1. Normally more than 90% fertilization can be obtained from this procedure (Chao et al. 1977).

For the conventional two-step freezing method, some preliminary experiments were performed using a programmable alcohol bath with a maximum cooling capacity of -2 °C per min and a minimum temperature of -65 °C. Most experiments were performed later in a programmable freezer (KRYO 10 Series III, Planer Biomed, England).

Various protocols have been examined with different cryoprotectants, cooling rates, holding times and seeding temperatures. The optimized protocol that yielded a survival rate of 78 ± 8% of the control value was as follows. Late morula-stage embryos were equilibrated in 2M DMSO plus seawater for 10 min at 25 °C. Embryos were pelleted by slow centrifugation and were aspirated into 0.5 mL straws. The straws were placed in the programmable freezer and cooled at -1 °C per min from 0 to -12 °C. Manual seeding was accomplished by gripping the straw with a pair of tweezers cooled in liquid nitrogen (LN_2). The straws were held at -2 °C for 5 min allowing equilibration after seeding. The embryos were cooled at -2 °C per min to -35 °C and allowed 5 min for equilibration before quenching in LN_2 for storage. Embryos were thawed by rapid warming of the straws in a water bath at 28 °C. After removal from the straws, embryos were directly placed in seawater to unload DMSO.

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Annotated Bibliography of Developments in the Last Decade

Since publication of this book in 2000, more studies have been reported to address cryopreservation of eggs, embryos, and larvae in oysters. Species such as the Pacific oyster *Crassostrea gigas*, eastern oyster *Crassostrea virginica*, and pearl oysters *Pinctada fucata* are important economic species around the world.

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Fertilization Evaluation of Cryopreserved Sperm of the Atlantic Croaker

Jin-Chywan Gwo

Mature Atlantic croakers *Micropogonias undulatus* were captured by gill net at Port Aransas, Texas, during the spawning season (From October to December) in 1988. Males and females were chosen by their physical condition and the apparent quality of semen or eggs, regardless of fish age or size. Prior to handling, each fish was anesthetized with tricaine methanesulfonate (MS222). Application of gentle pressure on the abdominal region along the midline of male fish was used to extrude semen. Caution was exercised to prevent contamination of the semen with urine, seawater, blood, and fecal material. Stripped semen was placed on ice and within 1 hr was extended in a 1% NaCl solution supplemented with dimethyl sulfoxide (DMSO). The dilution ratio [(volume extender + volume of DMSO): volume semen] was 10:1. No equilibration was needed. The diluted semen was drawn into 0.25-mL straws (instruments de medicine veterinaire-IMV, L'Aigle, France). The straws were frozen either in a programmable freezer or in liquid nitrogen vapor (at 5 cm above the surface) for 10 min and were transferred to liquid nitrogen for long-term storage. The straws were thawed in a 25 °C waterbath and used for fertilization when liquid first appeared in the straw.

Females were injected with 10 μg of luteinizing hormone-releasing hormone analog (LHRHa) per 100g of body weight. Ovulation occurred 30 to 36 hr after a single injection at 22 to 25 °C water temperatures. Eggs were stripped and inseminated within 20 min with either fresh or thawed sperm followed by the addition of seawater, yielding a final ratio of about 104 sperm per egg. Four min later, an excess of seawater was added. The fertilization rate was estimated as the percentage of viable embryos just before hatching (about 24 hr after fertilization at 25 °C). Viability was determined by the color of the embryos. Clear eggs containing moving embryos were considered to be viable.

Annotated Bibliography of Developments in the Last Decade

Since the publication of this book in 2000, no reports were found that specifically address sperm cryopreservation in Atlantic croaker. However, one review summarized sperm cryopreservation in marine fishes.

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Production of Cyprinid Embryos for Cryopreservation Studies

András Dinnyés, István Magyary and Béla Urbányi

Experiments were carried out in a commercial hatchery at Dinnyés, Hungary in June-July 1996 (Dinnyes et al. 1996, 1998). Eggs and sperm of common carp *Cyprinus carpio*, silver carp *Hypophthalmichthys molitrix*, bighead carp *Aristichtys nobilis* and grass carp *Ctenopharyngodon idella* were obtained as the result of injection with a gonadotropin releasing hormone analogue (GnRH-a; [D-Ala^6,Pro^{9}NEt]-mGnRH$_{A,}$ Interfish Ltd, Hungary). In a series of experiments, eggs from one female were mixed with sperm of two males and used in every treatment group. Immediately after insemination, eggs were treated with Woynarovich-solution (3-g NaCl and 4-g carbamide in 1-L water) in order to prevent stickiness of the eggs and to improve fertilization. One hr after fertilization, a treatment with tannic acid (3 g in 1 L water) was applied with the same purpose. To enhance hatching, embryos of the herbivorous fishes were treated with alkalic protease (1:5000 dilution). Embryos in the developmental stages of morula (common carp 4 hr, bighead carp, silver carp and grass carp, 3 to 4 hr), half-epiboly (common carp 8 hr, bighead carp, silver carp and grass carp, 6 to 7 hr) and heart-beat (carp 32 hr, bighead, silver- and grass carp 24 hr) were treated. Common carp embryos required 3 to 3.5 d for total development in hatching jars at 24 °C. Embryos of grass carp and silver carp required 30 hr at 22 to 24 °C, while bighead carp embryos hatched within 24 hr at 24 to 26 °C.

Experiments on Chilling Sensitivity

Treatment groups consisting of 300 embryos in 5mL of water in 10 mL plastic test tubes were placed into an icewater bath at 0 to 0.1 °C for 1 hr. After the treatment, embryos were washed twice with room-temperature water and placed into hatching jars. Control embryos were manipulated in room-temperature water in a similar way and placed back into hatching jars (Dinnyes et al. 1998, Baranyai et al. 1997).

Experiments on the Toxicity and Protective Effects of Cryoprotectants

Groups of 300 embryos in 5 mL of 1 M DMSO, methanol, or glycerol or in 0.1 M sucrose solution in 10 mL test tubes were placed in a water bath at 0 °C. After exposure for 1 hr, embryos were washed twice with room-temperature water and were placed into Zug-jars (Dinnyes et al.1998).

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Annotated Bibliography of Developments in the Last Decade

Since 2000 only two articles have been published about short-term storage of carp eggs, and two other articles deal with the toxicity of cryoprotectants and chilling sensitivity. However, the cryopreservation of fish embryos has become an active topic since 2000 with emphasis in other species. A selection of review articles that discusses embryos cryopreservation is listed below.

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An Inexpensive Hatching Jar

Donald W. Glenn III

We used self-standing, one-piece, 3-L plastic beverage bottles for construction of hatching jars (Glenn and Tiersch 1997). Three 5.08-cm circles were cut into the upper third of each bottle at equal distances to drain overflow water (Figure 1). At opposite sides of the bottle, 2.54 cm above the circles, a 0.95-cm bit was used to drill 2 holes. These holes provided access with plastic tubing to the jar interior and minimized disturbance of embryos when using a siphon tube to collect dead eggs or samples. Three 7.62-cm circles were cut from 0.16-cm nylon mesh screen. These were placed over the holes on the jar body to screen the outflow of water while keeping eggs in the jar. Silicone sealant was used to attach the screens and was allowed to cure before use.

A 1.91-cm polyvinyl chloride (PVC) schedule 40 pipe, 38.10 cm long, provided a water inlet. The bottom end of this pipe required eight 0.64 cm notches placed 0.64 cm apart. About 6.35 cm from the intact (top) end of the inlet pipe, a 1.91-cm rubber gasket was placed on the pipe and adjusted according to jar depth and cap placement. A 2.54-cm circle was cut into the center of the bottle cap to secure the inlet pipe and gasket to the jar body. Plastic tubing (1.27 cm diameter) was placed about midway inside the inlet pipe (to provide oxygenated water). The other end of the tubing was connected to a water source such as a recirculating system with a valve to control the flow.

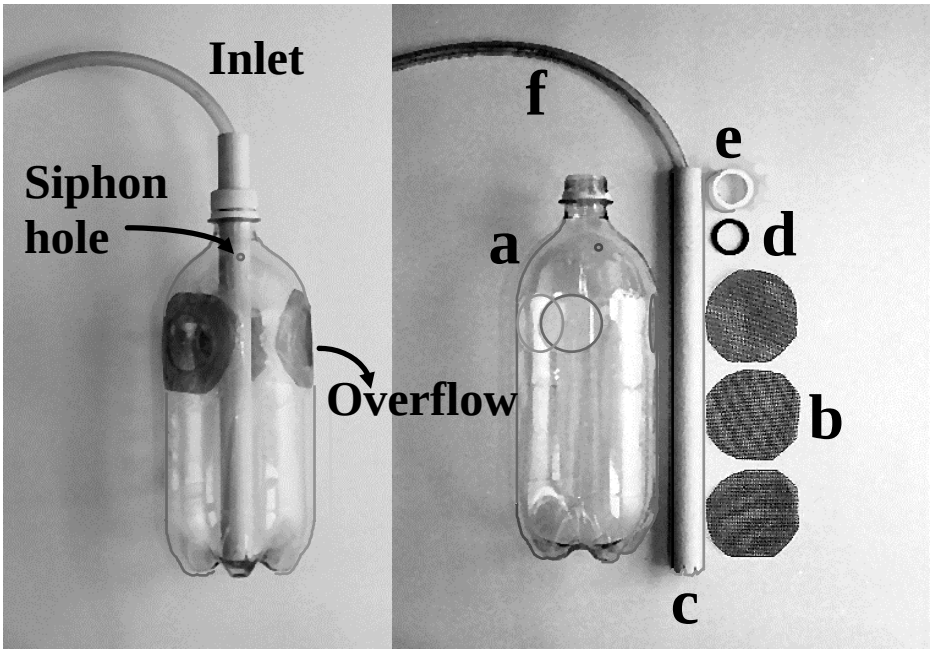

Figure 1. Assembled jar and components for the incubation of fish eggs: 3-L plastic bottle (a); circles of nylon screen (b); 1.91-cm PVC inlet pipe (c); 1.91-cm rubber gasket (d); original plastic bottle cap (e); plastic tubing of variable length for water inlet (f). Note the five protuberances (feet) on the base of the bottle which ensured even distribution of water. Figure reprinted with permission of the American Fisheries Society (Glenn and Tiersch 1997).

Glenn III, D. W. 2011. An Inexpensive Hatching Jar. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 719-720.

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Annotated Bibliography of Developments in the Last Decade

Improvements in equipment used for hatching of embryos and handling of larvae have been published in a number of journals since the original publication of this book. New designs that cater to specific species, or alterations in the general approach to incubating embryos and maintaining an environment for the newly hatched larvae continue to appear each year.

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An Inexpensive Rearing Tank

Mark C. Bates

Experiments with replicated treatments often require maintenance of small fish in separate containers. Systems constructed from disposable beverage bottles are inexpensive and can be tailored to a variety of applications (Bates and Tiersch 1995). All pipe and fittings described here for construction of rearing tanks (Figure 1) were of schedule 40 polyvinyl chloride (PVC) unless otherwise noted.

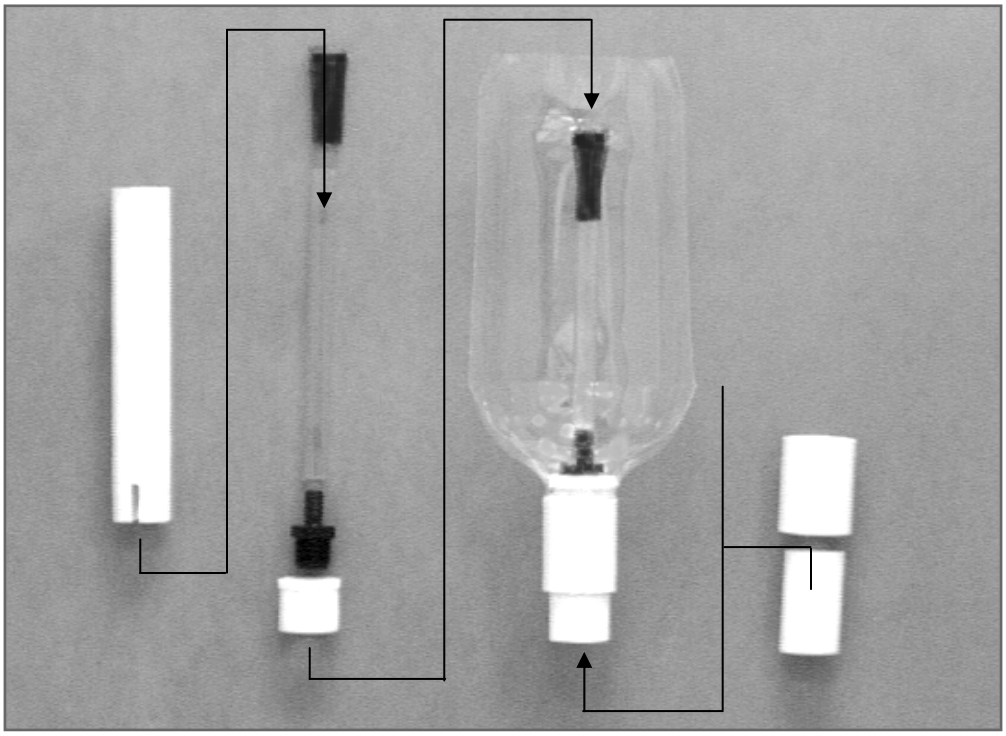

Figure 1. Construction of a rearing tank using a disposable beverage container, PVC pipe and fittings. Reprinted with permission of the American Fisheries Society (Bates and Tiersch 1995).

The neck of a 3-L plastic bottle (2.54 cm from the top) and the base were removed (5 cm from the bottom). A 2.54-cm x 1.27-cm female, normal pipe thread (NPT) reducer bushing was inserted into the bottle so that the 1.27-cm fitting was inside the bottle and the 2.54-cm fitting projected through the neck. Placement of the bushing in the bottle immediately after sawing allowed contraction of the cooling plastic to produce a tight fit around the bushing. Clear silicone sealant was spread around the 2.54-cm fitting already in the bottle and a 2.54-cm coupler was forced onto the bushing in the bottle. Silicone sealant was spread around the inside of the bottle to completely seal between the bottle and the bushing. A 1.27-cm male NPT x 0.95-cm barbed polycarbonate plastic fitting was placed into the 1.27-cm hole in the bushing. A 19 cm length of 1.27-cm clear rigid tubing was attached over the barbed end of the plastic fitting and sealed with clear silicone sealant. A 7.62 cm length of 2.54-cm diameter pipe was fitted into the 2.54-cm

Bates, M. C. 2011. An Inexpensive Rearing Tank. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 721-722.

coupler on the bottom to drain water from the bottle. A venturi drain was constructed from a 21.5 cm length of 2.54-cm diameter pipe with four vertical slits (2.54 cm high x 0.95 cm wide) cut in the bottom. A removable screen was made by cutting plastic window screen to wrap around the inner standpipe. The screen was sealed with clear hot melt glue along the side and the top.

For construction of tops, the base was removed (5.5 cm from the bottom) from a sec bottle, and a 2.54-cm hole saw was used to drill a hole for feeding in one of the five molded feet. A 0.95-cm drill bit was used to drill holes for air and water lines in two other feet. This modified bottom was placed on top of the each rearing tank.

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Annotated Bibliography of Developments in the Last Decade

The demand for low-cost approaches to handling and hatching of larval fishes continues to increase as more fish are utilized for biomedical research. Specialized apparatuses developed by companies are expensive and researchers continue to develop cost-effective alternatives within their respective facilities.

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Harper, C. J., B. M. Wrege, and J. J. Isely. 2010. Striped bass, *Morone saxatilis*, egg incubation in large volume jars. Journal of the World Aquaculture Society 41:633-639.

Heindel, J. A., Baker, D. J., K. A. Johnson, P. A. Kline, and J. J. Redding. 2005. A simple isolation incubator for specialized rearing of salmonid eggs and first-feeding fry. North American Journal of Aquaculture 67:13-17.

Performance of an Improved Temperature-Controlled Incubator

Donald W. Glenn III and Terrence R. Tiersch

Introduction

When dealing with artificial spawning of fish species, gametes must often be stored at specific temperatures to prolong fertilizing ability. Many studies have dealt with the issue of temperature and storage of sperm, eggs and embryos (e.g., Rothbard et al. 1996, Dinnyes et al. 1997). Storage of gametes can facilitate seedstock production and selective breeding, assist preservation of genetic diversity and expand research opportunities. Refrigeration of sperm also offers several advantages including hybridization and crossbreeding which can be performed in the hatchery. This particular study was part of a larger evaluation of the storage of eggs of koi carp *Cyprinus carpio* at different temperatures (Glenn 1998, Glenn and Tiersch 2002).

The purpose of this study was to modify general-purpose refrigerators to increase useable space and reduce spatial temperature variation to provide incubators for storage of gametes. Economics also played an important role. Commercially available incubators similar to the refrigerators in this study cost US $1000 or more. Other incubators with precise thermostat settings from 5 to 50 ^{o}C cost in the thousands of dollars. Information about the construction of incubators is limited, but a previous design was used as a guide for the use of an external hydraulic-action thermostatic controller (Tiersch and Tiersch 1993, Glenn and Tiersch 2000).

Modification Methods

A total of six incubators were constructed to maintain a constant temperature at different settings (0, 5, 10, 15, 20 and 25 ^{o}C). It required ~45 min to construct each incubator (Figure 1). Six dormitory refrigerators (0.06-m^3, Model 18SRCG, Gercor, Inc., Japan) were equipped with an external hydraulic-action thermostatic controller (Model 1609, White-Rodgers Division, Emerson Electric Co., St. Louis, Missouri, USA). This controller has a 2 ^{o}C differential setting that controls by opening and closing a refrigerant valve. Suction pressure controls the start and stop of the compressor through the motor starter. A small electric fan (115 V, Model 2412PS, Newark Electronics, Metairie, Louisiana, USA) was installed by exposing and separating the wires in the power cord for continuous operation. The interior door liner with built-in storage racks was removed by unscrewing existing tapping screws and replacing them with a 0.3-cm polyvinyl chloride (PVC) sheet (42 x 49 cm) to increase storage space. The original door gasket was removed carefully and replaced on the PVC sheet to ensure proper sealing of the door by using the original tapping screws in pre-drilled holes. In addition, plastic mesh (6-mm mesh size) was attached to the central shelf by use of plastic tie straps to provide a uniform surface for support of sample containers that would have otherwise passed between the shelf slats.

Installation of the thermostats involved exposing and separating the wires in the power cord. The 110-V (black) line was cut, the insulation stripped back, and the wire ends fitted with crimp-style terminals. The exposed portion of the cord was passed through the duplex connector at the bottom of the controller unit, and the line wire was connected in series with the controller. The exposed portions of the common (white) wire and ground (green) wire were left uncut and

Glenn III, D. W. and T. R. Tiersch. 2011. An Improved Temperature-Controlled Incubator. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 723-727.

tucked inside the controller, and the unit was mounted on the side of the incubator with sheet metal screws (e.g., Tiersch and Tiersch 1993). During installation, the capillary tube sensor was passed through an existing hole in the back of the incubator, and the sensing bulb was fastened with plastic tie straps under the center shelf. The original thermostat was not disabled, but was placed at a setting ("off") that would not interfere with the operation of the external controller.

Evaluation of Performance

Prior to installation of the door liners and electric fans in all six refrigerators, a study was performed to determine temperature variation with and without improved air circulation. The purpose was to evaluate the benefits of replacement of the door liner and use of the fan to remove temperature gradients. The desired temperatures were set using the factory-calibrated dial settings on the controller, and incubators were allowed to equilibrate for 24 h in a room held at 27 ^{o}C. To record temperature, the incubators were outfitted with three type-T thermocouples and a data logger. The thermocouples were placed in three areas: Area 1 was located middle, right, on the shelf beneath the freezer compartment; Area 2 was located on the upper left shelf, and Area 3 was located in the bottom left (Figure 1).

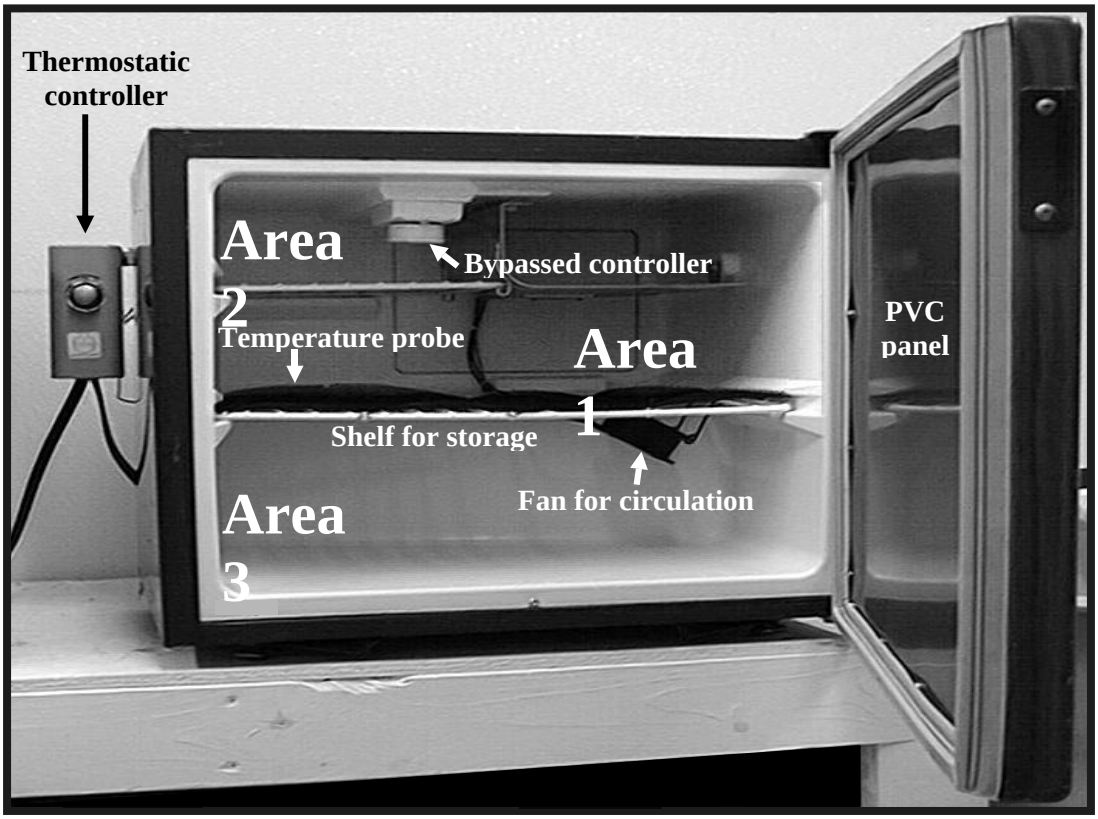

Figure 1. Front view of a modified incubator equipped with external hydraulic-action thermostatic controller mounted on the side. A sensing bulb and capillary tubing were inserted through a hole in back, and attached above the center shelf. The three areas indicated were used to measure temperature variation.

At a setting of 5 ^{o}C, the unmodified incubator showed significant spatial variation (P = 0.0001) in temperature (Table 1).

Table 1. Time and temperature relationships during a 5-hr test at a setting of 5 °C for modified and unmodified incubators constructed from dormitory refrigerators. The mean (± SD), and minimum and maximum values were obtained for three areas (Figure 1) within the incubators. The percentage of time is indicated when recorded temperatures were above, at, or below the set temperature during the test period. Area means sharing letters within an incubator type were not significantly different.

Area Monitored	Temperature (°C)			Deviation from temperature (% time)		
	Mean	Min.	Max.	Above	At	Below
Unmodified:						
1	1 ± 3^{d}	-3.4	5.9	12	0	88
2	6 ± 1^{e}	3.7	8.4	70	0	30
3	3 ± 2^{f}	0.2	6.9	27	0	73
Modified:						
1	5 ± 2^{a}	2.3	8.4	41	0	59
2	6 ± 2^{b}	3.4	8.7	60	0	40
3	5 ± 2^{c}	2.7	8.6	47	0	53

Although the temperatures in the three areas were stable, the lower areas were colder, and the coldest area (Area 1) was below the freezer compartment (Figure 2).

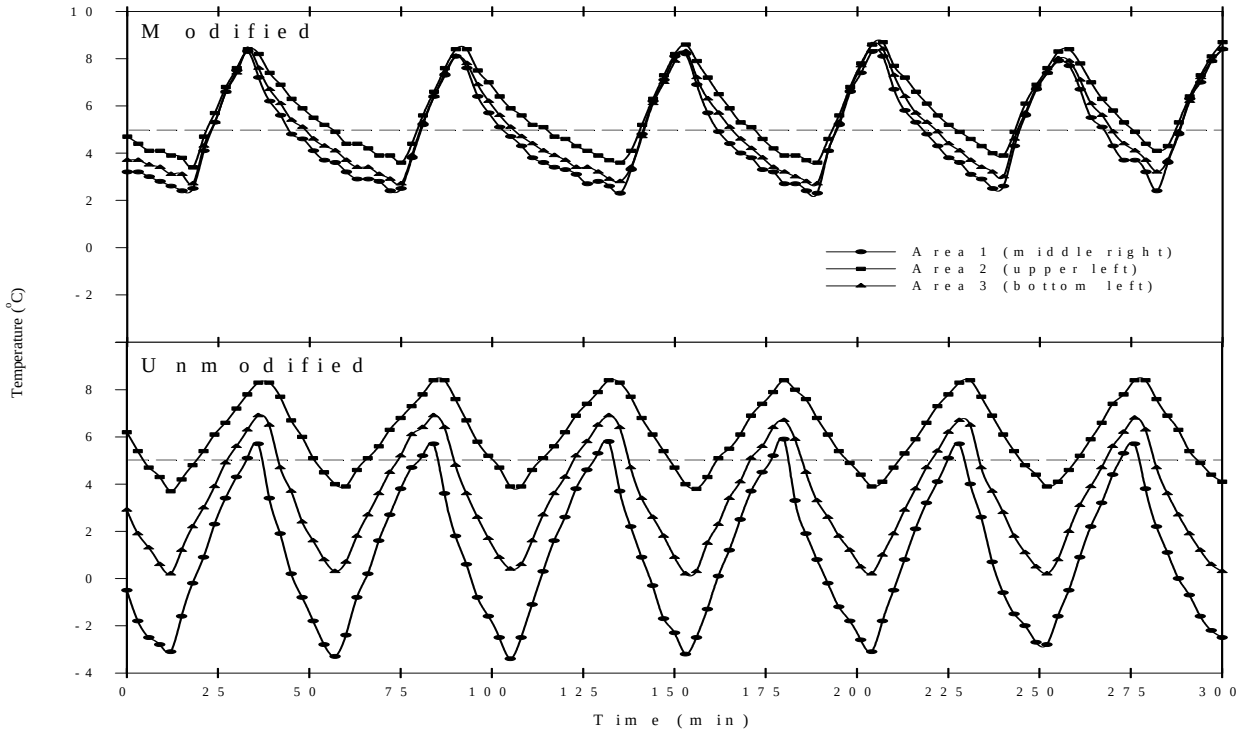

Figure 2. Comparison of spatial variation in temperature in modified and unmodified incubators set at 5 °C. Temperatures were recorded for three areas within the incubator: Area 1, middle, right (below the freezer compartment); Area 2, upper left, and Area 3, bottom left.

The values in Area 1 were below the set temperature for 88% of the test period and were at or below 0 °C for more than 2 h (43%) of the 5-h test period, posing the possibility of inadvertent freezing of samples held in this area. The results for the modified incubators were also stable in each area, although significantly different from one another ($P = 0.0003$). It was concluded that the modified incubators had improved airflow, reduced temperature variation and increased available space. Accordingly, all six incubators were modified with a PVC door liner and fan at a total cost of ~US $150 each in 2008 (including the cost of purchasing the used refrigerators from the LSU Housing Office at US $100 each).

Upon completion of the conversions, six desired temperatures (0-25 °C) were set using factory-calibrated dial settings on the controllers, and the modified incubators were allowed to equilibrate for 24 h. After equilibration, temperature inside the incubators was recorded for 5 h with the thermocouples positioned at the center of the middle shelf. The set temperatures agreed with the measured temperatures (Figure 3).

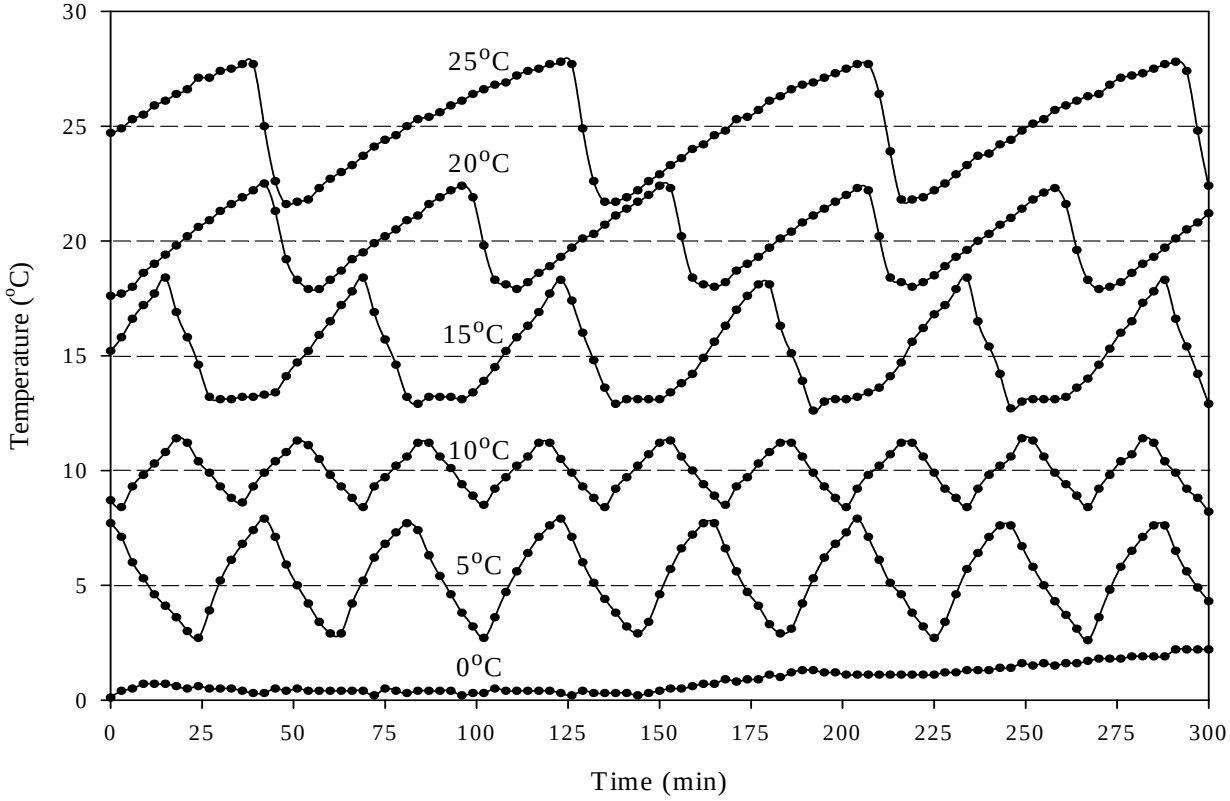

Figure 3. Temperature variation in modified incubators at six temperature settings (0, 5, 10, 15, 20, and 25 °C). A type-T thermocouple and data logger were used to record temperatures at the center of the middle shelf. Ambient room temperature (AT) was also recorderd.

The measurements for the six temperature settings were significantly different (Table 2). The time the temperatures were above or below the set temperature was approximately equal for the 5-h test period except for 0 °C which showed a longer cooling cycle having not reached temperatures below 0 °C.

Table 2. Time and temperature relationships during a 5-hr test at six temperature settings for modified incubators. The mean (± SD), minimum, and maximum values were measured at the center of the middle shelf (Figure 1). The percentage of time when recorded temperatures were above, at, or below the set temperature were calculated. Cooling rate refers to the periods of temperature decline, and cooling time is the percentage of the total 5-h period during which the incubators were actively cooling. Values sharing letters were not significantly different.

Set Temp.	Mean ± SD	Min.	Max.	Deviation from temperature			Cooling rate	Cooling time (%)
				Above	At	Below	(°C/min)	
25 °C	25 ± 2[a]	21.6	27.8	56	2	42	-0.57	12
20 °C	20 ± 1[b]	17.6	22.5	50	1	49	-0.31	30
15 °C	15 ± 2[c]	12.6	18.4	47	0	53	-0.33	18
10 °C	10 ± 1[d]	8.2	11.4	46	1	53	-0.18	54
5 °C	5 ± 2[e]	2.6	7.9	55	2	43	-0.25	45
0 °C	1 ± 1[f]	0.1	2.2	100	0	0	---	48

In all, these inexpensive incubators were useful in the storage of eggs of common (koi) carp while maintaining constant environmental conditions with minimal temperature variation, and providing sufficient space within each incubator for treatment replications. Incubators of this type would be useful for a wide variety of studies that require control of temperature, especially where numerous incubators are required for the purposes of experimental design. Approximately ten such incubators could be constructed for the cost of a single commercially available incubator (US $1,500).

Acknowledgments

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VIII. Genetics and Cryopreservation

Original Chapter: Bibliography Annotated by Authors

Genetic Consequences of Pooling of Sperm Samples

Andrzej Ciereszko, Jan Glogowski and Konrad Dabrowski

In hatchery practice, pooled milt from several males is often used for fertilization of the same batch of eggs. It is assumed that this procedure will ensure fertilization success because milt from a particular male may be deteriorated, and when used alone, could produce a low fertilization. However, it appears that even when individual males are capable of producing maximal fertilization rates when used alone, their contribution is not proportional when their milt is pooled with that of other males. In crosses made with pooled milt, individual males fertilized between 1% and 76% of eggs of chinook salmon *Oncorhynchus tshawytscha* (Withler 1988). The reason for these differences in fertilizing potency of spermatozoa of individual males was not explained. It is possible that these differences may arise from variance in sperm motility or from the presence of sites on the spermatozoan surface important for fertilization (Trummel et al. 1994). Further, individual male potency within a pool of milt may be modified by the timing of application of individual male semen (Gile and Fergusson 1995) or by storage time of pooled milt before fertilization (Withler and Beacham 1994). It is clear that uneven contributions by individual males can lead to loss of genetic variability within fish populations, when pooled milt is used for fertilization.

In numerous studies concerning cryopreservation of fish semen, it is a common practice to pool milt from many males. This yields a decrease in variability within experiments in comparison with higher variability when individual males are used. Pooling of semen also allows use of the repeated measures design for testing many cryopreservation variants (a common approach in cryopreservation experiments) using the same semen samples. Pooling of semen from several males is especially useful when males of a particular species produce small volumes of milt.

It has been recently found that the contribution of individual male rainbow trout *Oncorhynchus mykiss* to the fertilization success of cryopreserved pooled milt reflects their contribution in fresh semen (Babiak et al. 1998). In agreement with data for pooled fresh milt, the fertilization success of some males within cryopreserved pooled milt significantly deviated from proportional contribution. For this reason, restrictions concerning the usefulness of pooled semen for conservation of genetic variability also apply to cryopreserved milt.

In some cases, the cryopreservation process itself may influence survival of particular genotypes. Significant deviations from expected genetic proportions for offspring obtained by using cryopreserved semen of African catfish *Clarias gariepinus*, were found compared to proportions of offspring obtained by using fresh semen (Van der Bank and Steyn 1992). These deviations were related to specific effects of diluents, especially when glycerol was employed as a cryoprotectant, as compared to methanol or dimethyl sulfoxide. Another experiment showed that selective effects of cryopreservation on survival of spermatozoa of particular genotypes after thawing may also arise from non-optimal freezing rates (Van der Walt et al. 1993). However, when cryopreservation was performed in optimal conditions it did not influence genotype distribution in progeny as compared to fresh semen. Also, no selective effect of the cryopreservation process toward preference or elimination of particular males was found for rainbow trout (Babiak et al. 1998)

Ciereszko, A., J. Glogowski and K. Dabrowski. 2011. Genetic Consequences of Pooling of Sperm Samples. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 729-731.

In summary, when cryopreservation is optimally performed, it does not seem to be selective toward preference or elimination of particular genotypes of male gametes. Pooling of milt before cryopreservation, however, can cause uneven contributions of individual males in the progeny. Therefore, pooling of milt before cryopreservation may decrease conservation of genetic variability as compared to cryopreservation of milt of individual males. This should be recognized when sperm cryopreservation is used for conservation of genetic variability of fish.

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Annotated Bibliography of Developments in the Last Decade

After 2000 numerous papers regarding sperm competition have been published and their review is beyond the scope of this note. In our opinion it is important to notice that awareness has been raised regarding the long-term genetic consequences of hatchery-induced sperm competition (e.g. Campton 2004, Wedekind et al. 2007). Such consequences include the loss of genetic variation and induction of artificial selection. These problems must be kept in mind while using cryopreserved sperm for supportive breeding.

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Utilization of Androgenesis for Strain Recovery from Cryopreserved Sperm

Gary H. Thorgaard, Paul A. Wheeler and Robert D. Fields

Androgenesis is a chromosome set manipulation technique that allows individuals to be produced which have all of their nuclear gene inheritance from the male parent (Romashov and Belyaeva 1964, Purdom 1969). The procedure is not possible in mammals because both the maternal and paternal sets are needed for normal development (McGrath and Solter 1984). However, androgenetic individuals can survive in fishes and amphibians. Androgenesis has been used in fishes with the goal of generating homozygous, genetically uniform lines (Scheerer et al. 1991, Bongers et al. 1995, Young et al. 1996).

While methods for cryopreserving fish eggs have been unsuccessful, sperm cryopreservation is well established, as outlined in this volume. If only sperm is available from an endangered strain or species, androgenesis is the only available option for producing individuals with nuclear genes derived completely from the strain of interest (Stoss 1983). We will briefly outline how androgenesis could be used in strain recovery. To our knowledge, it has not yet been used for strain recovery for a conservation application.

Normal Procedure for Homozygous Androgenesis

Androgenesis involves inactivating the nuclear genes in the egg before fertilization. In fishes with large eggs or relatively opaque chorions, such as salmonids, ionizing radiation (e.g. gamma or x-rays) has most often been used (Arai et al. 1979, Parsons and Thorgaard 1984, Corley-Smith et al. 1996). More recently, it has been demonstrated that the nuclear genes of eggs from species with smaller, clearer eggs, such as common carp *Cyprinus carpiu* or Nile tilapia *Oreochromis niloticus*, can be inactivated with ultraviolet (UV) radiation (Bongers et al. 1994, Myers et al. 1995). This is advantageous because of the greater availability and convenience of UV sources. The availability of a range of suitable protocols for egg nuclear gene inactivation should facilitate the application of androgenesis to numerous species.

The fertilization of eggs with inactivated nuclear genes leads to production of haploid individuals. The essential challenge of androgenesis is the production of viable, vigorous androgenetic diploids. This is normally achieved by suppressing the first cleavage division, thus producing homozygous androgenetic individuals. However, producing individuals with two non-identical chromosome sets contributed by sperm is also an option.

If homozygous individuals are to be produced, a heat or pressure shock is applied to the fertilized egg before the time of first cleavage (Parsons and Thorgaard 1984). For example, in rainbow trout *Oncorhynchus mykiss* the optimal treatment time for eggs incubated at 10 °C appears to be about 5.5 hr after fertilization for pressure shocks and 3.5 hr post-fertilization for heat shocks (Palti et al. 1997). Viable homozygous individuals can be produced in significant numbers. In our laboratory, survival of androgenetic diploid rainbow trout to the initiation of feeding has averaged 3.5% (range, 0 to 22%) over 42 recent androgenesis experiments using eggs from 20 different females. The yield appears to be highly variable among females and likely correlates with egg quality. Sperm from the same males regularly give extremely different yields

Thorgaard, G. H., P. A. Wheeler and R. D. Fields. 2011. Utilization of Androgenesis for Strain Recovery from Cryopreserved Sperm. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 732-737.

of survivors depending on the egg lot used. It does not appear that previous inbreeding (removal of harmful recessives) increases the survival rate at this stage (Scheerer et al. 1986, 1991).

Reconstituting a strain with homozygous androgenesis would involve crosses among the first generation homozygous individuals that are produced (Thorgaard and Cloud 1993). Subsequent crosses among the sec generation heterozygous individuals would allow the production of genetically variable, outbred individuals (reconstitution of the strain) in the third generation. This approach could allow strains to be rebuilt using only cryopreserved sperm, with eggs being provided by other strains from the same species, or in some cases by closely related species. One improvement on this approach would be to sex-reverse some of the sec generation heterozygous fish produced by crossing among homozygous individuals into females (Hunter and Donaldson 1983). In a male heterogametic sex-determining system, these fish would normally be expected to be almost all males (resulting from crossing XX females and YY males), but by sex-reversing some XY individuals into females, both females and males would be available for producing the third generation progeny. Crosses of XY females and XY males would then yield 25% females in the third generation, which should be a sufficient fraction for strain propagation.

The homozygous androgenesis approach has some serious limitations. Probably the greatest limitation is the poor fertility of most homozygous females. In our experience, less than 10% of homozygous female rainbow trout have produced eggs that give viable offspring. This leads to difficulty in having enough eggs from homozygous XX females to produce the sec generation in the reconstitution plan. It also leads to the possibility of genetic bottlenecking in the reconstitution plan if only a few homozygous females contribute to the sec generation. Another potential limitation of androgenesis approaches is that mitochondrial DNA has been shown to be maternally inherited in androgenesis (May and Grewe 1993). Thus, if there are important differences in mitochondrial function among strains, those attributes would be lost during the androgenesis protocol for the strains being reconstituted from cryopreserved sperm. At this time, however, there is relatively little evidence to support the idea of major functional differences among fish mitochondrial types within a species (Danzmann and Ferguson 1995).

Atypical Androgenesis to Avoid Homozygosity

Because of the poor fertility of homozygous females, and to some extent the poor survival of homozygous androgenetic individuals in general, there has been some interest in the production of heterozygous androgenetic individuals. Such individuals could circumvent the fertility problems seen in the first androgenetic generation because they would not show inbreeding depression.

One approach for producing outbred androgenetic individuals is through the use of diploid sperm from tetraploids (Thorgaard et al. 1990, Arai et al. 1995). These efforts have demonstrated that survival can indeed be improved if homozygosity and the treatments inducing it are avoided and have allowed the sources of mortality in androgenesis to be assessed. However, using this approach for strain recovery would require that tetraploids be produced from the strains of interest before reconstitution, a significant limitation given the difficulty and time required for inducing tetraploidy in many species.

Another approach involves introducing two haploid sperm into the same egg. This does not have the limitation of requiring earlier generation of tetraploids, but has not yet been demonstrated to provide adequate survival rates for strain recovery. The main method used to

date has been to produce androgenetic diploids using fused sperm. Sperm can be fused to each other by several chemical treatments (Ueda et al. 1986) and such sperm have been shown capable of fertilizing eggs and yielding androgenetic progeny at a low rate (0.11%) (Araki et al. 1995). With improved survival this could be a viable approach for strain recovery. However, combining fusion with cryopreservation could be challenging, given that both procedures cause cell membrane damage, and the combination may be excessively damaging for fertilization.

Another possible approach involves fertilizing a single egg with two unfused sperm. Normally this is prevented by the micropyle of the egg, which acts as a block to polyspermy. However, it may be possible for a sec sperm to enter the egg if the chorion is digested. Potential means for dechorionation include the use of proteolytic enzymes (Hallerman et al. 1988) or hatching enzyme (chorionase) released during the normal hatching process. Chorionase can be collected by placing eggs in a small quantity of Ringer's solution at the expected time of hatching, followed by removal of chorions and other debris (DeMichele et al. 1981).

Experiments in our laboratory have demonstrated that a sec sperm could in some cases generally contribute to a surviving individual. In these experiments (Fields 1991), albino rainbow trout eggs were fertilized with sperm from albino male rainbow trout. After incubation in isotonic Ringer's solution and exogenous hatching enzyme for 45 to 60 min, eggs were re-fertilized with sperm from normally pigmented brook trout *Salvelinus fontinaliss*. This interspecific cross allowed easy detection of the karyotypic contribution of each parent species. A proportion (13%) of the embryos that survived to hatching was pigmented; three individuals were karyotypically confirmed triploids, and at least five were diploid-triploid mosaics. When the experiments were repeated using albino rainbow trout eggs and sperm, followed by hatching enzyme treatment and re-fertilization with pigmented rainbow trout sperm, five of the resulting progeny were pigmentation mosaics. Two of these mosaics survived for a considerable period (18 mo), suggesting that only a portions of the genetic complement of the two males were permanently incorporated. The use of irradiated eggs in such experiments might result in normal fertilization and development, because the mosaicism observed in the above experiments may be due to formation of tripolar spindles in the egg.

Is Androgenesis a Feasible Option for Strain Recovery?

Given the poor yields of viable and fertile offspring to date, androgenesis is not a preferred option for strain recovery. Survival rates have been too low with all of the "two sperm" approaches for these to be considered practical for real-world efforts at this time. Tetraploid approaches are impractical because of the need for prior production of tetraploids. Survival rates with homozygous androgenesis are more reasonable, but the poor fertility of homozygous females is a significant obstacle for propagation. Using cryopreserved sperm in crosses with closely related strains is a more practical option for reconstitution at this time. However, if that option is not available (e.g. no acceptably close strains are available) and pure strain recovery is deemed sufficiently important, a recovery plan based around homozygous androgenesis could be pursued with some chance of success.

Acknowledgments

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Annotated Bibliography of Developments in the Last Decade

Since 2000, much progress has been made in the area of androgenesis. Recently, androgenetic sturgeon were produced with cryopreserved sperm. The prospect of further success of this technique presents a valuable tool in conservation and line recovery. Research has also been conducted regarding biological effects of androgenesis.

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Isolation of DNA from Cryopreserved Fish Sperm

Lynn Pittman-Cooley and Terrence R. Tiersch

Introduction

As cryopreservation of fish sperm becomes increasingly applied, it creates new opportunities for genetic study of fishes. However, methods for the isolation of DNA from cryopreserved fish sperm have received only limited study (e.g. Cummings and Thorgaard 1994). Procedures for cryopreservation of fish sperm involve the use of cryoprotectant molecules and specific cooling rates to control the formation of ice crystals and the level of dehydration in cells during freezing. It is important to evaluate the effect of cryopreservation procedures on the purity and yield of DNA from fish sperm with respect to the long-term value of samples stored in germplasm repositories.

Cryopreserved sperm can be used for artificial spawning, and genetic analysis of frozen samples would be useful in marker-assisted broodstock selection for genetic improvement of aquaculture species. Cryopreserved sperm allows conservation of genetic resources in endangered species and control of genetic diversity in artificial propagation programs. Identification of rare alleles in frozen sperm could provide genetic markers for monitoring of stock enhancement programs in wild fishes. Isolation of DNA from cryopreserved sperm offers verification by genetic analysis of the source of sperm from high-value fish such as koi carp *Cyprinus carpio* that can be worth thousands of dollars apiece based on coloration and markings.

Therefore, this study was designed to develop procedures for DNA isolation from fish sperm cryopreserved for use in fertilization (Pittman-Cooley and Tiersch, 1999). Our objectives were to: 1) evaluate the effect of cryoprotectants, freezing rate, and storage temperature on the purity and yield of DNA isolated from cryopreserved sperm of channel catfish *Ictalurus punctatus*; 2) evaluate utility of the isolated DNA for use with the polymerase chain reaction (PCR), and 3) evaluate DNA isolation procedures for use with cryopreserved sperm of other fishes.

Sperm Collection and Refrigerated Storage

Sperm were manually stripped from four species of fish: spotted seatrout *Cynoscion nebulosus*, black drum *Pogonias cromis*, koi carp, and a federally listed endangered species, the bonytail chub *Gila elegans*. Because sperm cannot be stripped from channel catfish, testes were surgically removed and crushed. Sperm were suspended in Hanks' balanced salt solution (HBSS) prepared with reagent-grade chemicals (Sigma Chemical Company, St. Louis, Missouri, USA) (Tiersch et al. 1994). Because fish sperm become motile when diluted in water, the osmotic pressure of solutions was measured by vapor pressure osmometer (model 5500, Wescor Corp., Logan, Utah, USA) and the osmolality was adjusted to levels (Bates et al. 1996) that prevented activation of sperm in the freshwater species (300 mOsmol/Kg) and marine species (200 mOsmol/Kg) studied. For estimation of sperm motility, 2 µL of sperm were activated with 20 µL of distilled or salt water, and percent motility was estimated using dark-field microscopy (100-X). Only sperm samples with >50% motility were used for study.

Pittman-Cooley, L. and T. R. Tiersch. Isolation of DNA from Cryopreserved Fish Sperm. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 738-742.

Cryopreservation

Sperm samples from channel catfish (0.5 mL) were stored fresh at 4 °C (without cryoprotectant), or frozen at -20 °C (without cryoprotectant) or at -196 °C (with or without cryoprotectant). Samples receiving cryoprotectant were suspended in 10% solutions (final concentration) of DMSO (Sigma), glycerol (Sigma), or methanol (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and frozen at in 0.5-mL straws (IMV International Corp., Minneapolis, Minnesota, USA) sealed with polyvinylchloride powder. An equivalent amount of sperm (1.6 x 10^7 cells) contained in testicular tissue (calculated as 8 mg by weight) was placed in screw-capped cryogenic tubes (2.0 mL) and plunged directly into LN_2 as a control treatment for storage without HBSS. Sperm were stripped from the four other species and frozen in 0.5-mL straws using procedures established previously for each species in our laboratory (e.g. Wayman et al. 1996, 1997) and were stored in LN_2. Sperm samples were thawed by placing straws directly into a water bath (40 °C) for 7 sec (Christensen and Tiersch 1996). Motility was evaluated within 5 min after thawing.

DNA Isolation

For isolation of DNA, sperm were lysed with 3% b-mercaptoethanol (Amresco, Solon, Ohio, USA) and 2% SDS (Sigma) at 37 °C for 30 min. Samples of DNA were extracted using a mixture of phenol, chloroform and isoamyl alcohol (25:24:1) (Amresco), followed by another chloroform extraction. Proteinase K (Amresco) was added to a final concentration of 100 µg/mL, and samples were incubated at 50 °C for 1 to 3 hr. The DNA samples were extracted with phenol and chloroform, extracted again with chloroform, precipitated by adding 0.1-X volume of 3M sodium acetate (EM Science, Gibbstown, New Jersey, USA) and 0.7-X volume of isopropanol (Mallinckrodt, Chesterfield, Missouri, USA). Samples were centrifuged at 10,000 x g for 10 min, and pellets were resuspended in 100 µL of TE buffer composed of 10 mM Tris (Sigma) and 0.1 mM EDTA (Amresco). All samples were suspended at the same final volume (100 µL), and therefore yields were compared using concentration values. Absorbance was measured for DNA samples at 260 nm and 280 nm to determine yield (concentration) and purity.

Polymerase Chain Reaction

Analyses were performed with primers developed from channel catfish DNA, targeting the CH4 exon of the channel catfish gene encoding the constant region of the immunoglobulin M heavy chain (Thongpan et al. 1997). Primers were designed from the published DNA sequence (Wilson et al. 1990), and the expected size of the band amplified by PCR was 303 base pairs (bp). DNA samples were amplified using a Model PTC-100 thermal cycler (MJ Research, Watertown, Massachusetts, USA). Reaction mixtures (100 µL) consisted of 0.5 mg template DNA, 200 mM of each dNTP, 0.3 mM of the CH4 primers, 1.2-X reaction buffer, and 4 units (1 µL) of Vent® polymerase (New England Biolabs, Beverly, Massachusetts, USA). Samples were initially denatured at 95 °C for 5 min, followed by 30 cycles of: denaturing at 95 °C for 30 sec, annealing at 61 °C for 30 sec, and extending at 72 °C for 1 min. A final extension step of 5 min at 72 °C was performed. Products of PCR were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide (0.5 mg/mL), visualized on an UV transilluminator (Model 3-

3500, Fotodyne, New Berlin, Wisconsin, USA) and photographed. Polymerase chain reaction was not conducted on species other than channel catfish.

Statistical Analysis

Two separate analyses were performed. The first compared samples stored at three temperatures: refrigerated at 4 °C, or frozen at -20 °C or at -196 °C. The sec analysis addressed comparisons among the cryopreserved samples only (-196 °C storage). These treatments included samples stored in 10% DMSO, 10% methanol, 10% glycerol, and a control group to which no cryoprotectant was added. One-way analysis of variance (Excel 5.0, Microsoft Corporation, Redmond, Washington, USA) was used to test for differences in the amount or purity of DNA isolated. Duncan's multiple range test was used for pair-wise comparisons. The level for statistical significance was set at $P < 0.05$.

Results and Discussion

The concentration of DNA obtained from samples of fresh sperm was significantly higher than that obtained from the different freezing treatments. There was large variation overall in DNA concentration (range: 16 to 811 mg/mL), but even the samples with the lowest DNA concentrations (e.g. <50 mg/mL) yielded successful PCR amplification. Six of the lowest nine concentrations were from the groups cryopreserved in DMSO or glycerol. It remains to be determined if this is related to the observation that methanol is superior to DMSO and glycerol as a cryoprotectant for channel catfish sperm (Tiersch et al. 1994). The reason for the decreased yield from cryopreserved sperm in relation to fresh sperm is unclear, but sperm samples cryopreserved in 0.5-mL straws for use in artificial spawning yielded sufficient DNA for genetic analysis. This indicates that sperm frozen for breeding purposes can be used for genetic study without special preparation. There was no significant difference in purity among fresh and frozen samples regardless of freezing rate or storage temperature.

In PCR analysis, a DNA fragment of 300 bp in length was amplified from channel catfish DNA by use of the CH4 exon primers at a success rate of 90%. This success rate is based on the total number of DNA isolations examined in the study. It should be noted, however, that DNA from every fish was successfully amplified. Nucleic acid was isolated from cryopreserved sperm of the four other fish species with comparable yield and purity as that obtained from sperm of channel catfish.

Fish sperm is a readily available source of DNA and provides a number of benefits when cryopreserved. Unlike collection of blood or other tissues, collection of sperm is non-invasive in most cases. Sperm collection can often be incorporated into existing study programs, thus minimizing the cost of data collection. We have demonstrated in this study that use of various cryoprotectants and freezing temperatures allowed sufficient quantities of DNA to be isolated from cryopreserved fish sperm for genetic analysis. Successful isolation of DNA from cryopreserved sperm of rainbow trout *Oncorhynchus mykiss* has been reported (Cummings and Thorgaard 1994). Thus, isolation of DNA from cryopreserved sperm would seem broadly applicable among fish species, and offers the potential for analysis yr after sample collection.

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Annotated Bibliography of Developments in the Last Decade

Since 2000, four articles have been published directly on DNA extraction in cryopreserved sperm of aquatic species. However, a keyword search on DNA isolation resulted in >5000 articles. A keyword search on DNA and cryopreservation resulted in >500 articles.

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Preparation of Chromosomes from Cryopreserved Oyster Larvae

Quiyang Zhang, R. Paul Lang and Terrence R. Tiersch

Introduction

Unlike studies in biomedical fields, research in aquatic species is often hindered by the lack of pre-existing reagents, tools and methods. Research tools such as cell lines, DNA probes and tagged antibodies that are readily available, indeed taken for granted, for work in mice or humans, do not exist for aquatic species. This is especially true for genetic analysis of even commercially important aquaculture species. For example, it is well established that the quality of chromosome preparations is directly related to the quality of source material. For this reason, reproducible cell lines are often used as a source for high quality chromosomes. In mollusks such as oysters, there is no single cell type that possesses all traits required for use in chromosome studies (Zhang et al. 1999). Somatic tissues such as mantle and gill of adult oysters are available yr-round and provide chromosomes with minimal background. However, most adult tissues of oysters have low mitotic activity, and treatments for stimulation of mitotic activity in oysters are not available (Cornet 1993).

For such reasons, it has been extremely difficult to establish cell lines to support studies such as karotyping (Buchanan et al. 1999), and only a single cell line has ever been developed for mollusks (Hansen 1976). Meiotic chromosomes prepared from gonad tissue of oysters during spawning season are helpful for identification of individual chromosomes due to the presence of unique structures (chromomeres), however these chromosomes are not useful for karyotyping because of their diffuse and overlapping appearance (Figure 1). The embryos of oysters can provide actively dividing cells, and chromosomes prepared from these cells have morphology useful for mapping of gene location. Chromosomes at different phases of division can be found in these cells, and the elongated prophase and pro-metaphase chromosomes are most suited for application in high-resolution genome mapping (Table 1).

However, embryonic cells are only available during spawning season. Recent studies have shown that larvae of the eastern oyster *Crassostrea virginica* can be cryopreserved and remain viable after thawing (Paniagua-Chavez et al. 1998 and Paniagua-Chavez et al. this volume), circumventing the problem of seasonal availability of embryos. In channel catfish, we have used cryopreservation to store primary cultures of leukocytes for chromosome analysis after thawing (Zhang and Tiersch 1995). Our goal in this study was to develop procedures for obtaining chromosomes from cryopreserved larvae of the eastern oyster and to establish a yr-round source of material for preparation of chromosomes for physical genome mapping despite the lack of cell lines.

Materials and Methods

Cryopreservation

Eastern oysters were obtained from Grand Isle, Louisiana, and were maintained in an indoor recirculating system until use (Buchanan et al. 1998). Fifteen ripe male and female oysters were used for production of larvae by artificial fertilization which were

Zhang, Q. P. R. Lang and T. R. Tiersch. 2011. Preparation of Chromosomes from Cryopreserved Oyster Larvae. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 743-747.

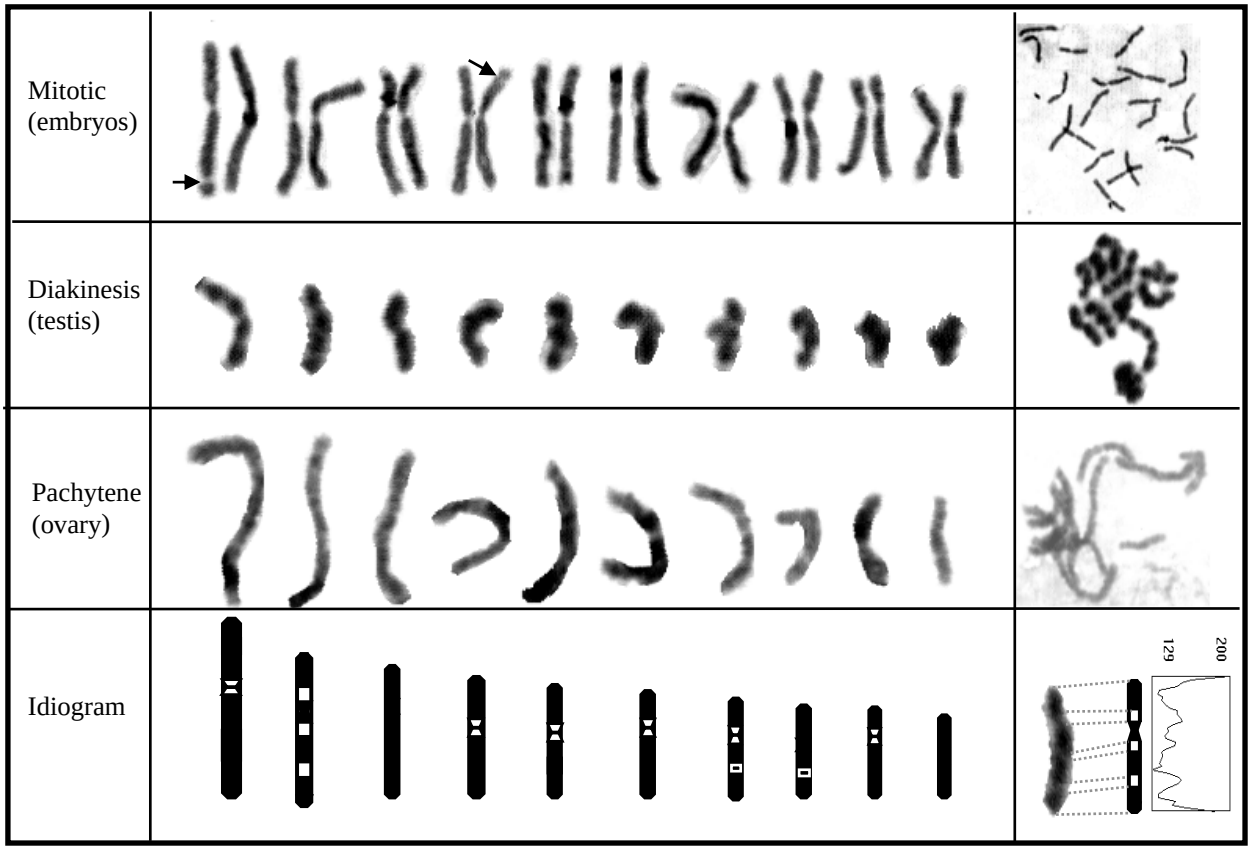

Figure 1. Karyotypes developed from different tissues of the eastern oyster. Arrowheads indicate secondary constrictions. The insets to the right for mitotic, diakinesis and pachytene chromosomes were the original spreads used for karyotyping. To the right of the idiogram is a demonstration of the method of microdensitometry (Zhang and Tiersch 1998). Reprinted with permission of the National Shellfisheries Association (Zhang et al. 1999).

frozen according to the methods described by Paniagua-Chavez et al. (1998) (Paniagua-Chavez et al. this volume). Larve were held at room temperature (~21 ºC) in 12-L buckets of artificial sea water (ASW) at 15 ppt until most had reached the trochophore stage (7 to 11 hr after fertilization). The larvae were concentrated and incubated for 15 min in ASW containing 10% propylene glycol, were frozen in 5-mL macrotubes at a rate of -2.5 °C per min to -30 ºC, and were stored in liquid nitrogen (LN_2) for at least 2 wk before thawing.

Preparation of Chromosomes from Frozen Larvae

Samples were thawed at 70 °C for 15 sec, were rinsed in ASW (15 ppt) for 30 min and were incubated in 0.01% colchicine (final concentration) for 40 min. The larvae were rinsed and incubated in 0.075 M KCl for 40 min, were fixed 3 times for 30 min each using cold methanol and acetic acid (3:1), and were stored in this fixative overnight. Chromosomes were prepared on glass microscope slides using standard air-drying procedures.

Table 1. Suitability of chromosomes derived from different tissues of the eastern oyster for cytogenetic analysis and physical genome mapping.

Characters	Tissue			
	Embryo	Gill (or mantle)	Testis	Ovary
Ploidy	2N	2N	1N, 2N	1N, 2N
Availability	spawning season	year-round	spawning season	spawning season
Division stage	mitotic metaphase	Mitotic metaphase	mostly diakinesis	mostly patchytene
Spreads per slide	>30	<5	*	*
Elongation	long	Short	intermediate	very long
Dispersal	separated	Separated	less separated	Overlapping
Resolution	high	Low	high	Highest
Centromeres identified	yes	Yes	no	No
Chromosome bands	no	No	yes	Yes
Chromosome pairs identified	7	3-5	10	10
Potential for mapping	suitable	not suitable	suitable	Suitable

* Cell division dependent on season.

Results and Discussion

The general morphology of chromosomes obtained from cryopreserved larvae (Figure 2a) was not different from that obtained from fresh larvae (Figure 2b). Survival and viability of the thawed larvae largely determined the success of chromosome preparations. Although optimal conditions for incubation after thawing remain to be determined, this study indicates that mitotic activity of trochophore larvae was recovered within 110 min after thawing (the time until fixation).

In previous studies, embryonic cells of the eastern oyster were found to be the best choice of source material for cytogenetic studies (Zhang et al. 1999). However, the limitations of using embryonic cells included seasonal availability and poor visibility of chromosomes due to adherent materials. This study demonstrated that cryopreservation is an effective tool for overcoming the first problem. We also found that chromosomes prepared from trochophore larvae were not obscured by adherent materials as seen for embryos.

Cryopreservation of oyster larvae has provided standardized test animals for analysis of chemical and biological pollution (McFadzen and Cleary 1994). Clearly cryopreservation of gametes, embryos and larvae can open the door to significant advances in a wide array of research areas. Thus, as stated elsewhere in this volume, the value and utility of cryopreserved samples is governed by labeling and record-keeping, especially for effective use in rapidly evolving fields such as molecular genetics where the range of tests is constantly expanding.

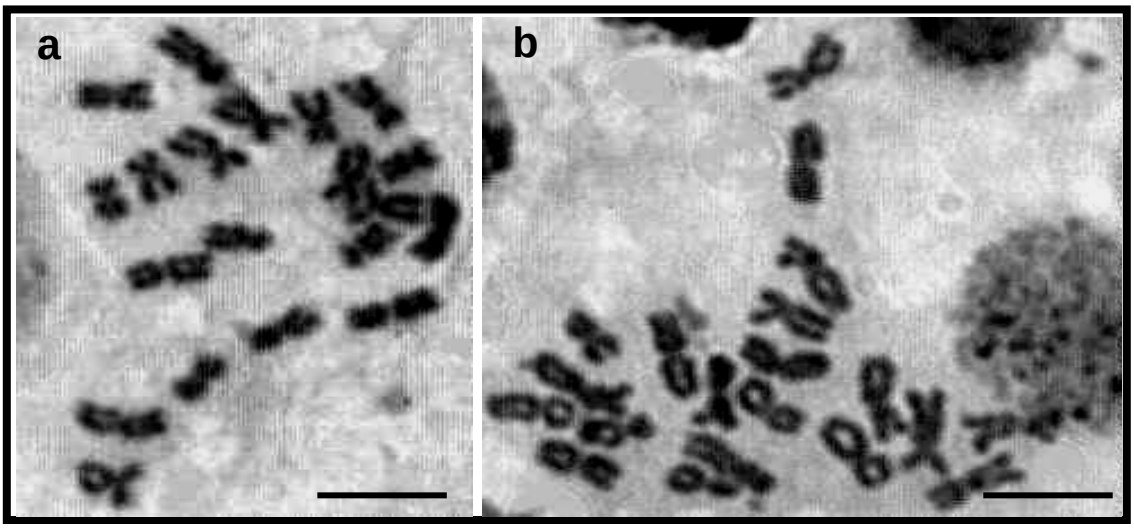

Figure 2. Mitotic chromosomes produced from cryopreserved (a) and non-frozen (b) trochophore larvae of the eastern oyster. Bar = 10 μm.

Acknowledgments

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Annotated Bibliography of Developments in the Last Decade

Few articles have been published on the subject of chromosome preparation in oysters since the publication of this book in 2000. In addition, few papers were found to have been published on chromosome preparation in oyster larvae or germplasm.

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New Chapter

Intracytoplasmic Sperm Injection (ICSI) in Fish

Germán A. Poleo

Introduction

Microinjection is a useful technique for the study of cellular phenomena, allowing direct introduction of substances in specific areas of the cell that can lead to the measurement of internal and external parameters such as pH and electric potential. This technique has helped to uncover physiological events such as membrane depolarization and the structure and function of ion channels. The tools required for microinjection have also been used for nuclear transfer in fish (Shaoyi et al. 1991, Wakamatsu et al. 2001), in mammals (Wilmut et al. 1997), and in the production of transgenic animals (reviewed by Chan 1999). It has also yielded crucial information for the study of mechanisms of fertilization by injection of sperm directly into the egg cytoplasm, a procedure called intracytoplasmic sperm injection (ICSI). The first experiments reported using ICSI were accomplished in invertebrates such as starfish (Lillie 1914) and sea urchin (Hiramoto 1962, Dale et al. 1985). In the 90 yr since the first experiments, this technique has been incorporated in the assisted reproduction of livestock (Kolbe and Holtz 2000, Goto et al. 1990, Catt et al. 1996), pet animals (Cochran et al. 1998, Gomez et al. 2000), and humans (Palermo et al. 1992). However, despite its early development in aquatic invertebrates and regular use in humans and farm animals, ICSI was only first accomplished in fish in 2001 (Poleo et al. 2001).

ICSI in Zebrafish

Basically the same equipment used for ICSI in mammals (Payne 1995) was used in zebrafish *Danio rerio* (Poleo et al. 2001, Poleo 2002). However, the characteristics of zebrafish eggs necessitated modifications such as the sizing of the injecting and holding pipettes and the medium used to hold eggs and sperm.

Although 72 of 188 ICSI eggs were fertilized by injection of sperm through the micropyle, only 3 (2%) developed to normal fish (Figure 1). The obvious question was why did many of the fertilized embryos develop abnormally? Puncturing oocytes with a glass pipette can mechanically damage structures involved in fertilization and development such as chromosomes, spindle apparatus, cell membrane, and cytoskeleton. This could compromise development or result in a lack of fertilization. In mammalian species, some of these problems can be avoided by injecting in specific locations where damage is minimized. For this reason to avoid the

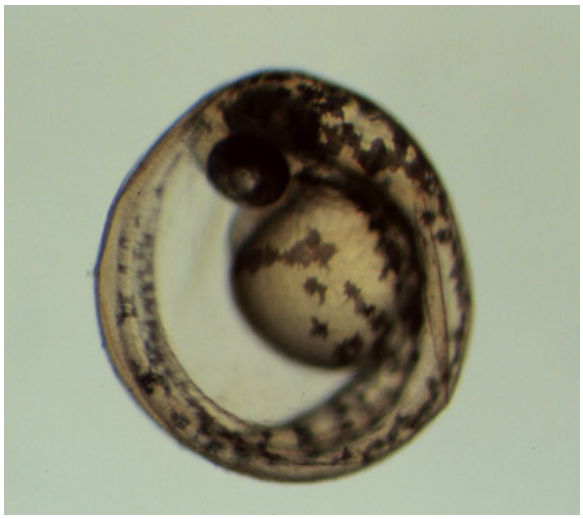

Figure 1. A young zebrafish named 'Poky': the world's first fish produced by ICSI.

Poleo, G. A. 2011. Intracytoplasmic Sperm Injection (ICSI) in Fish. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 748-753.

location of the maternal chromosomes, injection of sperm cells was directed to sites that reduced the possibility of cell injury (~100 μm from the micropyle) (Poleo 2002). Although the fertilization rate did not improve, the fact that embryos were produced by this method agrees with findings in medaka *Oryzias latipes* (Sakai 1961, Iwamatsu and Ohta 1978, Iwamatsu 1983), Pacific herring *Clupea pallasi* (Yanagimachi 1957), and rose bitterling *Rhodeus ocellatus ocellatus* (Iwamatsu and Ohta 1978) showing that sperm can fertilize eggs by entry at sites other than the micropyle in dechorionated eggs. Similar results were obtained in another study using injection of zebrafish eggs peripheral to the micropyle (efficiency ranging from 3% to 11%), but in this case, demembranated sperm nuclei were used in a technique called Sperm Nuclear Transplantation (Jesuthasan and Subburaju 2002).

Although zebrafish are important as ornamental fish and as research models, there was need to develop the technology for food fish, and thus ICSI was performed in Nile tilapia *Oreochromis niloticus* (Poleo et al. 2005b), and channel catfish *Ictalurus punctatus* (Poleo et al. 2001). Although the gametes of most fishes that spawn externally have similar characteristics, each species presented unique traits in features such as fecundity, egg size and morphology, localization of the sperm entry site, and mechanisms of gamete activation. These characteristics dictated the approaches that were used for each species during ICSI.

ICSI in Nile Tilapia

Nile tilapia eggs are ovoid and vary in size from female to female. This variation has been correlated with the size of the fish, with longer females producing larger eggs (Jalbert and Zohar 1982, Myers and Hershberger 1991). Eggs from Nile tilapia varied from 1.5 mm to 2.5 mm necessitating that the holding pipettes be made individually for each batch of eggs (between 100 μm to 300 μm in diameter) (Poleo et al. 2005b). It was observed that sperm injection in smaller eggs (~1.5 mm) was easier because handling during injection and observation of the animal pole was simpler. Nile tilapia proved to be a useful model for the application of ICSI. The spawning characteristics of these fish permit a reliable supply of eggs from individually housed females (Myers and Hershberger 1991). The size and morphology of the eggs facilitated the injection procedure, and the easy manipulation of embryos and sacfry led to greater survival after ICSI. However, as seen for zebrafish, ICSI in Nile tilapia yielded a low fertilization rate (9%) and a high frequency (83%) of abnormal embryos that may have been the result of chromosomal damage. The reasons for the low rate of fertilization and the production of abnormal fish remain unknown. As suggested for zebrafish, it is possible that the pipette damaged structures involved in sperm or chromosomal movement.

ICSI in Channel Catfish

The difficulties for injecting channel catfish eggs were not the localization of the micropyle ,or in the time the eggs could be held without losing fertilization capability (2-3 hr in Hanks' balanced salt solution) (Tiersch et al. 1994), but rather in the adhesive characteristic and the large size of the eggs which necessitated use of low magnification making sperm observation more difficult. Of the three species of fish described in this chapter, only channel catfish eggs were adhesive. This adhesiveness was an obstacle for ICSI as it hindered manipulation. However, to overcome this, culture dishes where eggs were held during the injection procedure were pre-coated using a commercially available solution of silicon (Sigmacote®, Sigma

Chemical Corp. St. Louis, Missouri, USA), and the tools used to manipulate eggs were coated with vacuum grease, including the holding pipette. The morphological structure of the egg and the large size of the micropyle facilitated the localization of the micropyle.

In contrast to zebrafish and Nile tilapia, injection of 188 channel catfish eggs yielded no fertilization. One possible reason for the lack of fertilization was that channel catfish eggs were not activated because no sign of activation was seen after injection. In channel catfish the mechanism of activation has not been studied, however changes in the chemical and morphological characteristics of the chorion are observed when the eggs come into contact with hypotonic water, and perhaps sperm (Grizzle 1985).

Future work in fish will need to address many of the same questions that researchers have addressed when performing ICSI in mammals, such as egg activation, sperm nuclear decondensation and migration, centrosome arrangement, and cell injury. In mammals and echinoderms it is known that the cytoskeleton of the egg plays an important role in sperm binding, sperm movement, and embryonic development. Disruption of cytoskeletal structures involved in fertilization could interfere with developmental processes. More basic studies are needed to clarify where disruptions can and do take place.

The Potential Application of ICSI in Fish

The need for germplasm repositories has encouraged researchers to develop techniques to preserve gametes and embryos for extended periods of time. The most common and effective method is cryopreservation, but the procedure can cause damage to sperm cells impairing motility, reducing the probability of fertilization. However, using ICSI, the injection of a single cryopreserved non-motile sperm cell can produce fertilization, as was shown by injection of cryopreserved Nile tilapia sperm into oocytes which triggered embryonic development to the blastula stage (Poleo et al. 2005a) or by injecting into zebrafish oocytes demembranated sperm that had been quick frozen without cryoprotectant (Jesuthasan and Subburaju 2002). Alternatives to cryopreservation of sperm cells that have been suggested include the use of solvents for sperm storage (Katayose et al. 1992) and lyophilization (freeze-drying) of sperm (Hoshi et al. 1994, Wakayama and Yanagimachi 1998, Kusakabe et al. 2001, Kaneko et al. 2003, Ward et al. 2003, Liu et al. 2004, Kusakabe et al. 2008). Unlike cryopreservation, sperm that have been chemically stored or freeze-dried are conventionally “dead” because they lose motility and membrane integrity, although the sperm nucleus can maintain genetic integrity when injected.

Intracytoplasmic sperm injection can be used for reconstitution of desired lines of fish and the production of small broodstock populations that can be used to produce large numbers of offspring. This approach could have application in restoring endangered or extinct stocks by injection of preserved sperm cells into irradiated eggs from a related species to produce androgenesis (all-paternal inheritance of nuclear DNA) (Purdom 1969, Parsons and Thorgaard 1985), or be used for the production of polyploid fish by injection of more than a single sperm cell, or for the generation of interspecific hybrids that would not be possible by other means.

Other applications could be in the production of transgenic fish. One problem in gene transfer in fishes is the occurrence of mosaicism (incomplete incorporation of the transferred DNA in various cells or tissues). A new approach has yielded stable and uniform gene transfer in zebrafish (Jesuthasan and Subburaju 2002) by injecting demembranated sperm cells incubated with linearized DNA into oocytes, producing non-mosaic transgenic fish without the need for breeding and growing foundation stocks.

Regardless of the potential use of ICSI, little has been done since the first fish was obtained with this technique in 2001. This will probably change in the near future due to the increasing importance of aquaculture around the world and the increased habitat destruction and overfishing that creates great need to acquire techniques useful to restore imperiled species.

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IX. Programmatic Developments of Germplasm Repositories

Ecological and Genetic Considerations for Collection of Gametes from Wild Fishes

Owen T. Gorman

Introduction

Gametes are collected from wild fish populations most often for use in propagation programs, but gametes may also be used to conserve genetic resources and conduct genetic studies. Propagation is an important component for most fishery management programs, including commercial stocks, sportfish or rare and endangered fishes. Gametes from wild populations may be used for propagation of wild stocks, captive broodstock development, stock improvement, development of new strains, stocking recovery programs and sportfish stocking programs, or they can be put into long-term storage to safeguard the genetic resources of imperiled populations.

Knowledge of the distribution, biology, ecology and genetics for species from which gametes are to be collected is important for developing strategies and criteria for collection of gametes and for formulating management and propagation programs. Wild populations of interest are often located in distant areas or in unexplored locations. Collection of gametes from rare and endangered fishes is made more challenging because of their reduced populations and protected legal status. Populations may be small and found in remote areas or consist of few individuals dispersed over a wide area. The best way to ensure success in obtaining suitable gamete samples from wild populations is for propagation biologists to work together with ecologists and geneticists that are engaged in monitoring and research programs. These fishery biologists know the locations and gear types needed to capture the fish, what types of habitats to sample, and how to identify their sex and the time of yr the fish will likely be in spawning condition.

Gamete collection from stocks of rare and endangered fishes needs to be integrated into a framework of ecological studies and monitoring programs. This is done to reduce impacts on these populations and to increase the success and efficiency of gamete collection. A principal objective of recovery programs for endangered fishes is to obtain sufficient ecological and genetic data from which to develop management strategies. Critical information needs include life history, ecology, population genetics, habitat requirements and reproductive biology. Detailed study plans must be developed and submitted to regulatory agencies to obtain permits to conduct studies and collect gametes and other tissues. In many cases, endangered fish are tagged and the identity of the gamete donors is known. Thus, much life history and ecological information is collected along with each gamete sample.

Field studies and monitoring of endangered fishes represent one component of recovery programs for endangered fishes. Propagation of captive broodstocks and rearing of fish in hatchery facilities for stocking in management areas is often necessary for recovery to progress. Also, the genetic characteristics of populations from which gametes are collected is relevant to improvement of captive broodstocks and propagation of genetically appropriate fish for stocking. Gamete collection from wild populations for use in propagation provides integration between field and hatchery components of recovery programs. The use of gametes from wild populations

Gorman, O. T. 2011. Ecological and Genetic Considerations for Collection of Gametes from Wild Fishes. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 755-759.

for propagation in hatcheries increases the genetic diversity of the fish to be stocked. Gamete samples can be stratified over a number of populations and habitats to increase the genetic diversity of the gametes collected. When collected within a carefully designed sampling program, gametes can provide a valuable source for information on population genetics. This information is useful in formulating management plans to maintain genetic resources for wild populations, developing broodstocks and in identifying desirable genotypes for recovery stocking programs.

Razorback Sucker Research

I will now provide two brief examples where collection of gametes has been integrated into existing field research and monitoring programs for endangered fish. The first example, the Razorback sucker *Xyrauchen texanus* is an endangered fish of the Colorado River in the southwestern United States. The largest remaining wild population is located in Lake Mohave, a mainstem reservoir on the Colorado River below Hoover Dam. For more than 6 yr, a diverse group of biologists integrated gamete collection into an ongoing, long-term monitoring program of razorback suckers in Lake Mohave (Tiersch et al. 1997, 1998). Critical monitoring information collected included population structure, migration patterns, population estimates, reproductive success and recruitment of new individuals. Research integrated into the monitoring program included studies of habitat use and spawning ecology, fish health, population genetics, collection of gametes and cryopreservation of sperm. Management actions later incorporated included genetic augmentation of captive broodstocks and production of fish from wild stocks for recovery stocking programs. Executing this complex monitoring and research program required detailed study plans and coordinated work by numerous scientists.

Close collaboration between field and propagation biologists was critical to the success of the razorback sucker propagation program at the Willow Beach National Fish Hatchery located on Lake Mohave, Arizona. Every spring during 1994 to 1999, the biologists convened at the hatchery to conduct coordinated field and propagation studies for about 10 d each yr. Field biologists were knowledgeable of when and where to find ripe fish for gamete collection. For example, because spawning activity peaked at night, sampling was conducted after dark at known spawning areas. The field biologists collected sperm from wild fish and transported these samples and gravid females to the hatchery for use in propagation efforts. After the females were stripped, field biologists returned them to the place of capture. Field studies identified two spawning phenotypes that were used in propagation, those fish that spawned along quiet lake margins over clean gravel and those fish that migrated 10 to 50 km upriver to spawn in swift water over loose gravel bars. Field biologists were also able to provide unique identification numbers for each fish, capture location, habitat, approximate age, length, weight, parasite load, general health, reproductive condition and yield of gametes. This information was recorded on data sheets in the field and transferred to the propagation biologists at the hatchery facility along with sperm samples and gravid females (Figure 1). The team approach to razorback sucker propagation was successful. During 6 yr of collaborative research, monitoring, and propagation efforts, more than 500,000 fry were produced from wild fish and were used in stocking programs throughout the Colorado River basin. Additionally, considerable knowledge about sperm collection, storage and cryopreservation, and propagation of razorback sucker was acquired (e.g. Carmichael et al. 1996, Tiersch et al. 1997, 1998) as well as spawning ecology (e.g. Gorman et al. 1999).

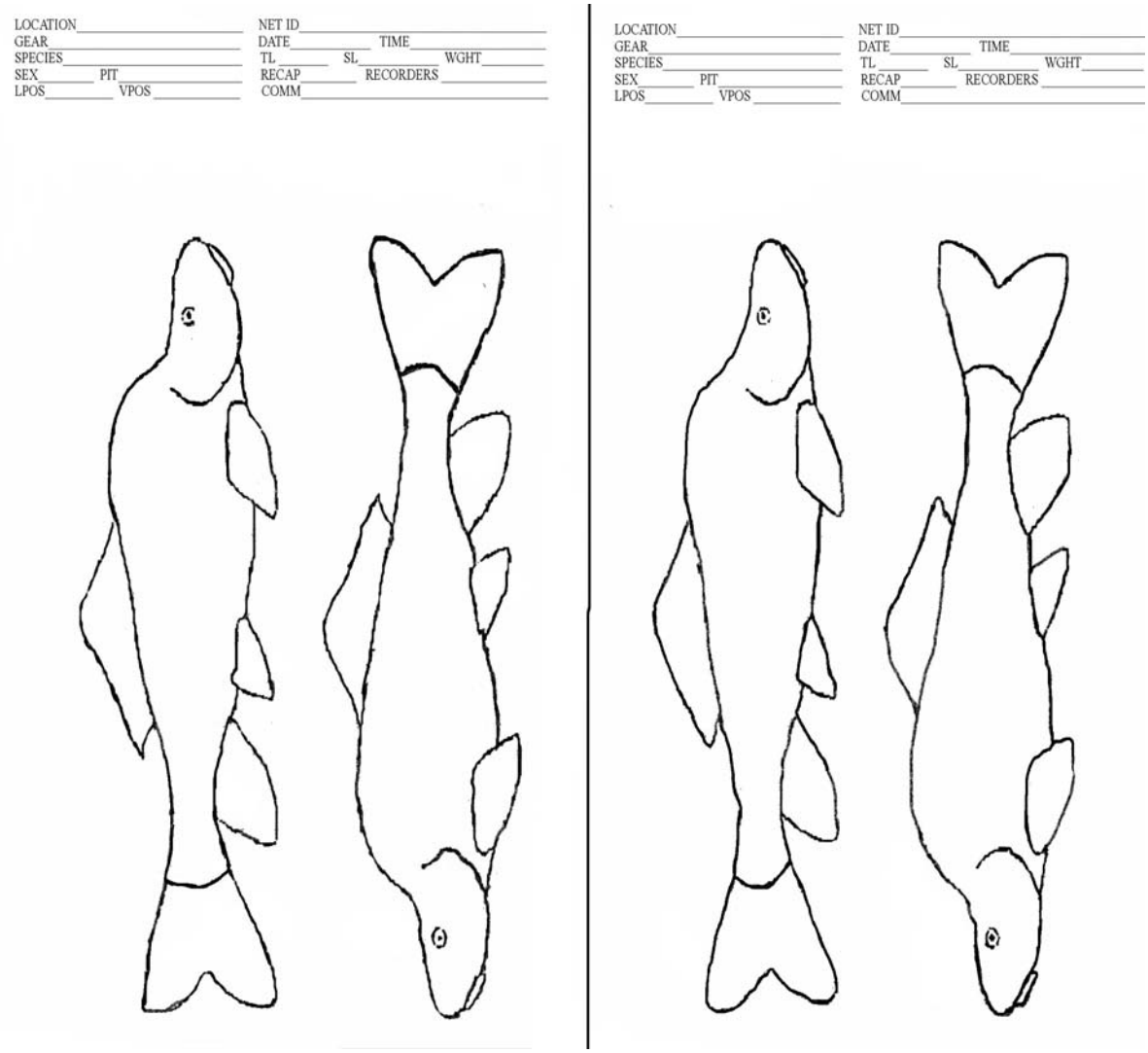

Figure 1. Sample field data sheet for razorback sucker studies in Lake Mohave. Information recorded includes date, time, collection site, sex, length, weight, and identification number. Additional information includes reproductive condition, yield of sperm, parasites, presence of nuptial tubercles (TB), coloration, abrasions and general fish health (e.g. cataracts and blindness). Data sheets accompanied sperm samples and gravid females to the hatchery where propagation studies were conducted.

Humpback Chub Research

The sec example, the Humpback chub *Gila cypha* is another endangered fish of the Colorado River in the southwestern United States. The largest remaining population is located in the Grand Canyon in Arizona. Access to populations located in remote areas of the Canyon require transport by helicopter or by rafting more than 100 km downstream through whitewater and rapids. Preliminary studies to determine feasibility of collection and cyropreservation of sperm were performed in the Little Colorado River gorge in spring of 1995 by a team of propagation and field biologists. This effort was integrated into ongoing monitoring and field ecology studies of the species. As with the razorback sucker, field biologists were familiar of

when, where and how to catch ripe humpback chub for gamete samples. The study was successful in developing techniques for collection and cyropreservation of humpback chub sperm and samples of frozen sperm were successfully transported from the Canyon to a university research facility more than 3,000 km distant. The results of ongoing monitoring and ecological research have identified isolated populations of humpback chub throughout the Canyon and field biologists have proposed using cryopreservation of sperm as a means to conserve the genetic resources of these imperiled populations. In 1998, field biologists collected several hundred humpback chub and transported them via helicopter from the Grand Canyon to the Willow Beach National Fish Hatchery, a distance of more than 500 km. These fish represent the first captive broodstock of humpback chub in almost 20 yr. Years of field study yielded detailed information of the spawning ecology of humpback chub (Gorman and Stone 1999). With the establishment of a captive broodstock, development of gamete collection methodologies, and findings from ongoing field studies and monitoring, all of the ingredients necessary for a successful propagation program using gametes from wild populations are in place.

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Annotated Bibliography of Developments in the Last Decade

The importance of ecological and genetic considerations for collection of gametes is emphasized by the updated references for this chapter, which include an online book on sharing and conserving the world's aquatic biodiversity (Greer and Harvey 2004), and eight reviews on topics such as conservation genetics of aquatic species, freshwater fish biodiversity, conservation of aquatic resources through use of freshwater protected areas, global biodiversity decline in freshwater and marine fisheries, and threats, conservation and prognosis of suckers (Catostomidae) in North America. Research on the two endangered fishes used to illustrate the integration of gamete collection into existing field research and monitoring programs for endangered fishes has continued, especially for the razorback sucker, for which research has

been steadily reported throughout the last decade, with a total of 31 publications. However, research on the humpback chub has been sparse, with four publications referenced here.

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Development of Databases for Germplasm Repositories

Harold L. Kincaid

Introduction

The primary purpose of a gene bank is to preserve the gene pool of unique populations by protecting them from extinction, loss of genetic variability (inbreeding) and dilution due to interbreeding with unrelated populations (hybridization). Gene banks may be used to achieve goals including: 1) restoration of threatened or endangered populations; 2) expansion of threatened or endangered populations; 3) broodstock improvement through genetic modification of a targeted population, and 4) research for information and technology development. Fish population gene banks can be of two types: live animal banks based on living animals maintained at multiple locations to provide a continuous source of progeny, or tissue gene banks based on gamete, zygote or somatic tissues held in cryogenic storage for later retrieval to produce progeny. Currently in most species, the male sperm cell is the tissue most reliably placed in cryogenic storage, retrieved and used for reproduction to perpetuate the source population. Throughout this discussion, the term "population" is used to refer to all interbreeding fish "groups" (i.e. strains, stocks or broodstocks), wild and cultured, that are essentially closed to introductions from other groups.

The gene bank concept is designed to preserve target populations by "freezing-in-time" the total gene pool until sufficient natural habitat has been restored to support that population. In light of the extended time period (decades) required to restore habitats and the large number of populations that may comprise the gene pool of a single species, huge amounts of information must be collected to characterize each population and its natural habitat. The only way all this information can be managed effectively for gene bank managers and gene bank users is with a well designed computerized database.

Fish Databases

Computerized databases for fish population information have not been available to fisheries personnel until the last few yr. Kincaid et al. (1994, 1998a, 1998b) developed the National Fish Strain Registry (NFSR), an information database to characterize populations of managed wild and cultured fish species throughout the United States[*]. The database contains information on broodstock origin, life history, reproduction, habitat preference, cultural performance, genetic analysis and current management and aquaculture applications. Database information can be viewed as a series of pre-formatted tables or as specific data subsets using the database query function. The NFSR contains characterization information on managed populations from the families Salmonidae (inland trout), Ictaluridae (catfish), Acipenseridae (sturgeon) and Polyodontidae (paddlefish). This is a dynamic database designed to accommodate expansions as new data become available. Froese and Pauly (1997) described the FishBase97 database, an international database containing information on over 17,500 fish species

[*] Copies of the National Fish Strain Registry database are available from the author at the following address: USGS, Research and Development Laboratory, NFSR, Library, R.D. #4, Box 63, Wellsboro, Pennsylvania 16901 USA or email: hkincaid@usgs.gov.

Kincaid, H. L., 2011. Development of Databases for Germplasm Repositories. In*: Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 760-769.

throughout the world. Both databases contain extensive information on multiple species that can be used to assist fisheries personnel with management decisions.

Gene Bank Management Information

Extensive information is required to manage the broad range of activities that are essential for an operating gene bank such as population sampling procedures, sample identification, tissue processing, cataloging, information retrieval, quality control and sample disposition. Gene pool preservation requires that a sufficient number of individuals be collected to effectively sample the total allele pool and to achieve gene and genotype frequencies representative of the source population. Typically, population sampling is accomplished by collecting gametes from a large, random sample of individuals from the target population. The actual number of individuals necessary to achieve this goal is debatable, but the criteria most commonly used is the smaller of a pre-determined percentage of the total population (10 to 100%) or an upper limit number of individuals (100, 400 or more) that sampling theory indicates is sufficient (Kincaid 1983, Allendorf and Ryman 1987, Ryman 1991). Once milt is collected, a sample identification system is applied to ensure positive identification of each sample during all handling procedures (processing, storage, monitoring and retrieval operations). A quality control program is needed to monitor and ensure that high viability is maintained throughout the storage period. A milt replacement program is needed to systematically replace samples that deteriorate during storage, are used in the quality control monitoring program, or are removed for breeding and research programs. Extensive records are required to document inventory changes as materials are added or removed from the gene bank (e.g. dates, reason for change and adjusted inventory).

Gene bank information is divided into two primary categories: 1) gene bank operations information to catalog and manage the stored tissues, and 2) gene bank user information to characterize the source population and determine the most effective applications for stored tissues. The database must include the information needed to accomplish the following ten functions: 1) sample and population identification procedures; 2) collection procedures; 3) sample processing procedures; 4) sample storage and retrieval operations; 5) quality control monitoring; 6) inventory maintenance; 7) information retrieval; 8) milt replacement program; 9) milt discard procedures, and 10) user applications information.

The decision to preserve a specific population gene pool requires the collection of baseline information to determine population uniqueness and to characterize the traits causing that uniqueness. Baseline information includes population name, location, range and distribution, genetic characterization, breeding history, life history, habitat preferences, and behavioral characteristics. Species with no history of cryogenic preservation will also require preliminary studies to determine species-specific cryogenic processing and storage procedures before gene banking operations can begin. Population characterization and validated storage and retrieval procedures are information essential to guide day-by-day operations and to ensure reliable long-term preservation of each population gene pool.

The population information needed by gene bank users are essentially the population characterization data, stored milt quantity and quality data and the effective population size represented in the preserved milt. Information on population origin, breeding history, source habitat parameters, life history traits, genetic analysis and performance characteristics in culture and natural habitats are needed to uniquely define each population. Information on fish sampled by date, location and number collected are used to determine sampling completeness and the

realized effective population size of the preserved gene pool. This information allows gene bank users to match available populations to the management situation and to identify the most appropriate applications. The first priority for milt from threatened and endangered populations will be restoration and expansion of the original population. Only milt that is surplus to the restoration or expansion objective should be available for other breeding and research applications.

Planning the Gene Bank Informational Database

A database is a collection of data organized in a systematic order conducive to rapid searching and retrieval of desired information subsets. Design of the information management database should be one of the first steps in planning a gene bank because the design process requires the manager to systematically think through every gene bank operation. Database design involves the identification of specific data elements and development of a data structure that is time and space efficient. Essential information includes data needed by gene bank managers to facilitate decisions on management and research applications where preserved milt will be used.

The initial data element or trait list is developed by answering, as completely as possible, a series of questions similar to those shown in Table 1. The questions are divided into two categories, with Category 1 representing the information needed for gene bank operations and Category 2 the additional information needed to assist user groups to determine the priority applications for milt stored from each population (i.e. restoration or enhancement, broodstock improvement and research programs). Each question should suggest specific traits that fulfill information needs; these traits are recorded on the trait list, separately for each question. The preliminary trait list is complete when a set of traits is identified that provides the targeted information for all of the questions. The list is then reviewed and multiple recordings of the same trait are deleted. The sec step is identification of the gene bank function (listed above) when information on each trait is collected. The third step is identification of the gene bank function or functions when information for each trait will be used to guide or support a decision. As new traits are identified during this process they are added to the trait list. When these three steps are complete, the composite information is placed in a table to identify the relationships between individual data element needs and uses (Table 2). Knowledge of these relationships will guide database development to achieve a structure that is efficient in terms of computer storage space and response time for data summary, query and retrieval operations.

Representatives of all potential user groups must participate in the initial trait identification process to ensure that all known information requirements are included when the database structure is designed. User groups should include federal and state

Table 1. Suggested questions to guide development of the initial trait list for a new gene bank information database. Actual questions for any given gene bank information database may vary depending on the specific objectives, applications and species preserved in that specific gene bank.

Category 1. Gene bank operations and Management	
Information to describe	**Example traits or characteristics**
a. Population or sample identification system?	Population identifier, sample identifier, fish identifier, ampule or straw identifier, etc.
b. Population sampling plan?	Population size, number of collection sites, number of individuals collected per site, total number of individuals collected, etc.
c. Milt field handling procedures?	Date spawned, sample and fish identification, capture method, spawning method, anesthetic, individual fish measurements, milt holding method, milt quality measurement, etc.
d. Milt transportation system?	Cooling and freezing methods, shipping temperature, transit time, transportation mode, etc.
e. Source population characteristics?	Origin, breeding history, life history, reproductive traits, behavioral traits, habitat preference, performance in cultural and natural environments, etc.
f. Source population genetic characteristics?	Analysis method, allele frequencies, heterozygosity, distinctive characteristics, etc.
g. Source fishery characteristics?	Location, water body type, approximate size, water quality, food sources, predator species, etc.
h. Gene bank processing procedures?	Milt quality, additives, diluents, dilution rate, freezing procedure, storage unit type, storage unit size, storage location, etc.
i. Milt gene bank function	Individual sample sub-division method, sub-samples per individual retained for: 1) allocation plan; 2) gene pool archive; 3) quality control monitoring; 4) breeding applications, and 5) unanticipated need reserve, etc.
j. Quality control and monitoring procedures?	Milt testing schedule, test sample selection procedure, testing methods, fertility rate, motility rate, etc.
k. Milt replacement procedures?	Replacement criteria, replacement schedule, minimum inventory, population sampling procedure, etc.
l. Milt discard procedures?	Discard criteria, minimum viability and fertility levels, disposal method, discard documentation, etc.
m. Restoration or enhancement procedure?	Program identification, date removed, milt quality, percent fertilization, number progeny produced, progeny disposition, procedure for choosing milt samples for program, restoration or enhancement program results, etc.
n. Broodstock improvement procedure?	Program identification, date removed, milt quality, percent fertilization, number progeny produced, progeny disposition, procedure for choosing milt samples for program, broodstock improvement results, etc.
o. Research program procedure?	Program identification, date removed, milt quality, percent fertilization, number progenyproduced, progeny disposition, procedure for choosing milt samples for program, research program results, etc.

Table 1. Continued

Category 2. Gene bank user information (in addition to Category 1 information). Users will include fisheries managers, hatchery managers, aquaculturists and scientists working with population restoration and enhancement, broodstock improvement and research projects

Information to describe	Example traits or characteristics
1. Source population characteristics?	Additional traits and characteristics of special interest to milt user: 1) origin, 2) source habitat parameters, 3) life history traits and 4) cultural performance traits, etc.
2. Source population status?	Threatened or endangered, effective population size, disease and health status, etc.
3. Source population genetic characteristics?	Analysis method, allele frequencies, heterozygosity, effective population size, genetic analysis, stocking history, etc.
4. Gene bank sample genetic purity?	Genetic analysis, population sampling procedure, gene bank sample identification methods, etc.
5. Milt availability throughout planned project?	Gene bank population milt inventory, gene bank milt replacement procedure, restoration and enhancement program milt use schedule, etc.
6. Source population genetic basis?	Effective population size, genetic analysis, stocking history, etc.

Table 2. Relationships between database functions, gene bank protocols, information collection, and information utilization by gene bank management and users of the stored milt in restoration and enhancement programs, broodstock improvement programs and research programs.

I. **Plans and procedures established to fulfill gene bank functions prior to the start of gene bank operations.**

1. Population and sample identification system
2. Milt field handling procedures (collection, processing and storage)
3. Population characterization traits
4. Milt processing procedures
5. Milt quality control and monitoring plan
6. Milt removal procedures for applications
7. Population sampling plan (milt field collection)
8. Milt transportation procedure
9. Source fishery characterization traits
10. Milt storage plan (number and size storage units per population)
11. Milt replacement plan
12. Milt removal procedures for disposal

II. **Flow of Gene Bank Database information.**

Operations	Database information collected during operation	Database information needed to conduct operation
1. Collection baseline characterization information	Population characterization information Fishery characterization information Fishery identification and name Confirm population sampling plan with modifications	Population characterization traits Source fishery characteristization traits Population sampling plan (milt field collection)

Table 2. Continued.

Operations	Database information collected during operation	Database information needed to conduct operation
2. Milt collection (field)	Number fish sampled per site Milt quantity (individual) Milt quality (individual) Fish measurement (individual) Document milt collection procedures	Population and sample identification system Population sampling plan Milt field handling procedures (collection, processing and storage)
3. Transport form field to laboratory	Document Transport procedures	Population and sample identification system Milt transportation procedure
4. Milt processing procedures	Measure milt quality Document milt additives used Document milt processing procedures Document freezing procedures Document milt storage allocations Establish milt inventory summary	Population and sample identification system Gene bank milt procedures Gene bank milt storage plan (number and size of storage units per population) Gene bank milt quality control and monitoring plan
5. Quality control program	Development schedule for monitoring Establish “minimum” viability for retention Document quality control test results	Population and sample identification system Gene bank milt quality control and monitoring plan
6. Milt inventory replacement program	Number fish sampled per site Milt quantity (individual) Milt quality (individual) Update milt inventory summary	Population and sample identification system Milt inventory summary Milt replacement plan Status of ongoing applications projects (approved) Future needs for approved applications projects
7. Milt discard procedures	Document milt discard procedure Update milt inventory summary Review need for replacement milt	Population and sample identification system Milt inventory summary Status of ongoing application projects Results of quality control programs Future needs for approved applications project Required “minimum” viability for retention
8. Milt used for restoration and enhancement programs	Document milt removed by program Review status of source populations (restoration and enhancement programs) Update milt inventory summary Status of ongoing applications projects	Population and sample identification system Milt inventory summary Milt replacement plan Milt removal procedures for applications
9. Milt used for broodstock improvement programs	Document milt removed by program Status of ongoing broodstock improvement program Update milt inventory summary Status of ongoing applications projects	Population and sample identification system Milt inventory summary Milt replacement plan Milt removal procedures for applications
10. Milt used for research programs	Document milt removed by application Review status of ongoing research program Update milt inventory summary	Population and sample identification system Milt inventory summary Milt replacement plan Milt removal procedures for applications Status of ongoing applications projects

fisheries management agencies, endangered species programs, agency and university researchers and commercial aquaculture interests. Inclusion of potential database users during the design phase facilitates identification of information needs before gene bank operations start and minimizes the need for later repeat sampling to collect missing information. In addition, it permits users to identify information formats most useful to them. Specific information in any given gene bank management database will vary depending on gene bank objectives, species biology, population variability and expected applications.

Components of Gene Bank Information Management Database

Once information needs and data relationships are defined, programming the gene bank information can begin. Data relationships in combination with desired data summary and output formats would guide the final database structure decisions. The organization of database components will determine the degree to which the database is "user friendly." Essential components of the gene bank information management database are discussed below.

Data Input Screens

Data entry is facilitated by input screens that mimic the data collection forms used for field and laboratory data collection. Carefully designed entry screens can improve data entry efficiency by reducing the number of missed entries and by facilitating rapid data verification

Data Edit Screens

The inclusion of data editing capability, along with edit screens that reflect the data collection forms, allows rapid retrieval of individual records and reduces the time required to add new information, change existing entries or perform data verification operations.

Records Review Capability

The capability to recall individual records and view the complete record allows the user to quickly review the status of populations and individuals.

Data Summary

Pre-programmed tables and graphs to summarize selected information for individual populations and across populations within species, allows the user providing a quick review of the information.

Query Capability

The capability to query the total database for information on specific traits designated by the user greatly expands information accessibility. Pre-formatted summary tables can provide the most commonly requested information, but many times users need information on specific data subsets that cannot be anticipated in summary tables.

Information Output Method

Database users need the option to view information on the computer screen, as paper printouts, or in data files that are exportable to other applications programs.

Data Entry and Edit Control

Database managers need to maintain control over data entry and editing operations and can do this in many ways. The most common method is a two-level password access procedure

that allows users to view database information without the capability to edit. Database personnel would have full access for data entry and editing operations.

Summary

Development of the information management database should be a high priority when establishing a gene bank. The gene bank information management database is essential because it is the management system that will catalog, summarize, query and retrieve information from the data set required to establish and operate the gene bank. When constructed to incorporate operating protocols and procedures, the database applications program can effectively manage all database functions such as data entry, data editing, information queries, sample disposal, records maintenance and records review. Close coordination between gene bank managers and potential user groups during database development will greatly facilitate the inclusion of all user information needs. Once the trait list is complete, the database can be constructed to calculate desired data analysis and provide information in output formats required by gene bank users.

Management relies on the database to schedule routine gene bank operations (quality control testing, sample identification, sample location, current inventory, etc.) and to support management decisions (milt sample replacement process, milt allocation to projects, etc). Fisheries managers using preserved milt will rely on the database for accurate information on source populations, milt quality, milt availability, current inventories, etc.

The gene bank information management database is, in a sense, the day-to-day gene bank manager. The database provides the basic information required to efficiently operate the gene bank and to effectively use preserved gene pools to manage approved programs: restoration and enhancement, population and broodstock improvement and research.

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Annotated Bibliography of Developments in the Last Decade

Updated references on database development for plant and animal germplasm repositories include publications on development of software (Genebank Information Management System) for germplasm database management, strategies for organizational and information technology framework and management support for creation of national gene banks, and one review article on international database systems for farmed animals, including a network of decentralized country biodiversity and gene bank databases (FABISnet databases), and molecular databases such as GenBank (NCBI, National Center for Biotechnology Information), EMBL-Bank (European Molecular Biology Laboratory-Bank), and DDBJ (DNA Databank of Japan). There were only two articles on database development for germplasm repositories for aquatic species. The most recent publication (Eugenio-Gonzalez et al. 2009) was on development of the Germplasm Bank of Aquatic species of Baha California (GBAS), GBAS information management system (GBAS-IMS), and GBAS-IMS database design. Another reference (Wayman 2003) centered on the development of a cryopreserved germplasm repository for aquatic species, with emphasis on sturgeon. For updates on the National Fish Strain Registry Database see *gcmd.nasa.gov/records/GCMD_brdlsc0005.html* ; and *www.lsc.usgs.gov/SPN.asp?StudyPlanNum=08028*.

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New Chapter

The Role of Cryopreservation in Integrating Genetic and Genomic Research Programs at USDA-ARS and the Advancement of Domestication in Aquaculture

Jeffrey Silverstein

Introduction

Aquaculture provides half of global seafood, with about 50 million tons grown worldwide in 2006. Half the seafood consumed in the United States comes from aquaculture, and about 85% of that amount is imported. Seafood demand in the United States and worldwide is steadily increasing due to population growth and increased consumption per person. But fish and shellfish taken from the ocean are already at or above sustainable levels, so any increase in seafood supply will have to come from aquaculture. The Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA) is committed to providing tools and resources required by aquaculture industries to meet the consumption demands of U.S. consumers. Through collaborations here and abroad, these efforts will result in sustainably raised seafood that is nutritious and delicious and supports human and environmental health.

The need for characterizing and preserving genetic variation is vital and this need is shared with resource agencies such as the U.S. Department of the Interior (including the Fish and Wildlife Service, and the U.S. Geological Survey) and the Department of Commerce (including the National Oceanographic and Atmospheric Administration, and the National Marine Fisheries Service). Yet beyond preservation of genetic resources, there is additional potential for cryopreservation to provide commercial aquaculture with avenues for genetic progress in domestication and agronomic performance. The use of cryopreservation of germplasm in research and development of aquaculture resources is in early stages. Nevertheless, cryopreserved semen has been used for example, to assist incorporation of new genetic variation into privately owned hybrid striped bass populations (Craig Sullivan, North Carolina State University, personal communication). Indeed, it is easier to ship frozen straws of semen instead of a sexually mature 25-Kg striped bass.

Overall, use of cryopreservation in aquaculture has been slow to catch on for a number of reasons including: a lack of readily available protocols for freezing and thawing (which is being addressed by publications such as this book), the early stage of development of most domesticated aquatic animal populations, and the related lack of well-defined elite males for cryopreservation. Aquaculture research at the USDA-ARS is directed at addressing genetic improvement and storage of germplasm of improved populations. The aquaculture sector is developing along patterns similar to the development seen for other agricultural animal industries. Principally management and nutrition have been improved upon, and over the next 10-20 yr, development of domesticated populations of fish and shellfish will increase along with reliance upon genetic predictors that will contribute to increased productivity and profitability. To accomplish this, greater emphasis will need to be placed on capturing phenotypic and genotypic measures that will facilitate genetic analyses, and enable positive genetic change. These capabilities have been developed in the dairy industry, and in the rest of this chapter, I will

Silverstein, J. 2011. The Role of Cryopreservation in Integrating Genetic and Genomic Research Programs at USDA-ARS and the Advancement of Domestication in Aquaculture. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 770-773.

describe ARS research activities that will facilitate further movement of aquaculture resources toward development of comparable capabilities.

Aquaculture Program Directions at USDA-ARS

Breeding programs at the ARS, several of which are administered in conjunction with university counterparts, are developing fast-growing fish and shellfish with enhanced disease resistance, fillet yield, and reproductive traits. These programs are based on traditional selective breeding practices, and are moving quickly to incorporate new information and approaches arising from genomics research. One priority of the ARS aquaculture breeding programs is to develop genomics libraries and bioinformatic tools for current and emerging farmed aquatic species and to incorporate that information into breeding research to enhance available germplasm. Data from phenotypic, nutrition, and health studies will feed back into genomics work and provide direct avenues to enhance production. The use of cryopreservation to make elite germplasm available over time and distance has not been well developed in aquaculture for several reasons (as stated above), including variability in fertilization success (e.g., Figure 1).

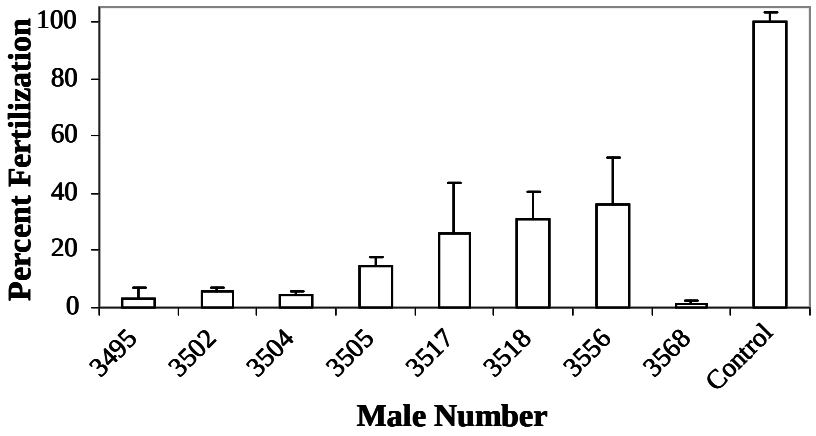

Figure 1. Variability in percent fertilization success (x ± SD) of cryopreserved semen samples of rainbow trout *Oncorhynchus mykiss* and fresh sperm control (from Silverstein et al. 2007).

Development of domesticated aquatic animal populations and well defined elite males will increase the demand for cryopreservation technologies and services. Aquaculture research at USDA-ARS is directing strong efforts at genetic improvement and germplasm storage for improved populations.

Lessons Available from Crops and Livestock

In plants, the use of stored germplasm to screen for agronomically valuable traits (e.g, rust or scab resistance) and subsequent introgression of those traits into production material has a long and successful record. In animal agriculture, especially aquaculture, it is not yet common to store cryopreserved germplasm. Because the generation interval in animals is longer than in plants, the definition and isolation of genetic markers for key traits is more difficult. There can

be a considerable performance drag associated with using germplasm that has not undergone selective breeding. In addition the offspring numbers (to screen for successful incorporation of selected traits) are lower than in plants, and thus in general introgression of specific traits has not been widely practiced in terrestrial animals. The greatest value of stored, cryopreserved germplasm has been for *ex situ* maintenance of genetic diversity, and for preservation of genetic material from individuals with superior breeding values. In aquaculture species, the potential to screen germplasm and identify traits for introgression into domesticated lines may be greater than for traditional livestock species. This is because offspring numbers are typically much greater than in terrestrial animals, and the majority of aquatic species do not have a long domestication history and offer greater opportunity for improvement.

Phenotypic Databasing

Recently, the storage of germplasm with databases of associated phenotypic data from large numbers of individuals has demonstrated value in animal agriculture. The rapid drop in the cost of DNA sequencing is revealing new opportunities for collection of genotypic information for animals with phenotypic records. Such databases, including genomic and phenotypic data will be requisite for employing marker-assisted and whole-genome selection methodologies that are being developed across animal industries (Meuwissen et al. 2001). The availability of DNA samples from individuals with phenotypic records was invaluable for the dairy industry to take advantage of the rapid drop in genotyping costs. Great focus on collecting and maintaining this type of information for aquaculture species is a key component for moving ahead in utilizing advanced breeding methods.

The 50-K SNP Chip

Cryopreserved sperm was a critical resource for developing predictions for dairy performance by correlating medium density single nucleotide polymorphism (SNP) marker arrays (such as the 50-K SNP chip) with animal performance. Genotypes were produced for 3,576 Holstein bulls with semen samples and phenotypic daughter records primarily from the years 1995 to 1998. Predictive equations were generated, and data from 1,759 bulls produced with cryopreserved sperm between 1999 and 2002 and the phenotypic records for the daughters were analyzed in combination. The reliability of prediction using genotypic data was improved (50% reliability with genomic data vs. 27% without genomic data) over pedigree information alone (the previous standard) (VanRaden et al. 2009). For comparable utility in aquatic species, it will be essential to define target populations and obtain substantial phenotypic and genotypic information across these populations to effectively use tools such as medium to high density SNP chips for genetic predictions.

The Need for Integration Across Programs

Although the value of semen cryopreservation for elite bulls has been recognized for decades, this new application of genomics has highlighted another value of germplasm collections – as an information reservoir. The need for aquaculture programs to generate phenotypic records especially on animals that are contributing to germplasm repositories must be emphasized. Generating the numbers of phenotypic records and genotypes to enable the kind of prediction accuracy achieved within the Holstein population will take considerable time, effort,

and coordination of resources. There is critical need to define and target key phenotypes and store these data along with the cryopreserved material.

Conclusions

The lack of a significant number of genotyped animals that have documented phenotypes for a variety of traits is and will continue to be an extreme limitation for further development and implementation of genomic tools in aquaculture and livestock industries. It is foreseeable that development and integration of databases or knowledge bases as described above could be a key direction and important contribution for animal germplasm repositories, including development of comprehensive repositories for aquatic species.

Acknowledgments

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New Chapter

The USDA National Animal Germplasm Program and the Aquatic Species Collection

Harvey D. Blackburn

Introduction

Diversity of genetic resources and the genetic variability within species are the raw materials by which the productivity of populations can be increased for food production. Genetic variation provides opportunities for increasing feed efficiency, growth rate, and conferring resistance to disease, among other traits. Due to the recognized importance of these resources, efforts nationally and internationally have begun to protect them (FAO 2007). Aquatic species are atypical among agricultural activities being only at the beginning of using management practices that alter gene frequencies for commercial benefit. Despite this, important trout, salmon, shrimp, and catfish populations have been developed for commercial use. An intended consequence of developing specific lines for commercial purposes is to make commercial lines more phenotypically uniform and less genetically diverse.

While selective mating has commercial benefit, it can have negative long-term consequences, the ramifications of which has been seen with Holstein cattle *Bos taurus* where nationally there are approximately 4 million head of this breed of dairy cattle, but the effective population size is only about 36 head. This remarkable contraction of Holstein genetic diversity took 40-plus years to accomplish, and the same situation could be created quicker with aquatic species due to the number of offspring a single pair of parents can produce and the relatively short generation intervals. Recent work suggests some genetic contraction may be occurring, with a report of relatively high F_{IS} values (inbreeding coefficient) and deviations from Hardy-Weinberg expectations for microsatellite markers in rainbow trout *Oncorhynchus mykiss* (Silverstein et al. 2004).

The dynamic situation described above is a call to action and a number of governments worldwide are responding to this situation by establishing national *ex-situ* or *in-situ* conservation programs. The objective for the remainder of this chapter will be to describe the structure and function of the National Animal Germplasm Program of the USDA Agricultural Research Service, which has the mandate to collect, preserve, and assess genetic resources of food and fiber producing animals in the United States of America.

NAGP Formation and Structure

Contraction of genetic resources has been of concern for portions of the livestock industry and public sector groups for some time, but it was not until 1990 that national legislation was passed that provided the US Department of Agriculture (USDA) with a mandate to conserve animal genetic resources. This legislation provided further impetus for public and private sector initiatives to explore necessary conservation actions (NRC 1993a). By 1999, USDA decided to initiate a livestock conservation program, through the newly formed National Animal Germplasm Program (NAGP). To assist in formulation of the new program the American Dairy Science Association sponsored, as part of their Discover Conference series, a

Blackburn, H. D.. 2011. The USDA National Animal Germplasm Program and the Aquatic Species Collection. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 774-779.

meeting in late 1999 (and again in 2004) with public and private sector participants to plan and initiate conservation activities. The meeting resulted in the formulation of six committees (aquatic species, beef cattle, dairy cattle, poultry, small ruminants, and swine) to assist in the identification and acquisition of germplasm and tissue for a genebank. The development and application of a genebank for livestock species was a relatively new activity for public sector organizations. However, the plant community has used genebanks as an integral component of genetic resource conservation strategies and breeding programs (NRC 1993b), demonstrating that a wide variety of germplasm can be collected and preserved.

Species Group Committees

The NAGP has a broad mandate considering the range of various animal species, geography, and technical disciplines required to execute its mission. As stated above, to accomplish its goals the program established six species group committees to provide input to the program concerning the collection of specific populations, cryopreservation practices and genetic considerations. A unique feature of each committee is the composition of its membership – each committee has members from industry, universities, and government agencies. This combination serves the program by ensuring that a wide range of populations are targeted for sampling. In addition, the committees bring a wide range of technical expertise and insight into the various industries and their sub-sectors which facilitates the acquisition of germplasm.

Necessary Supporting Technologies

Three broad categories of technology are needed to construct a functioning gene bank for animal genetic resources. These include: information systems, reproductive biology and cryopreservation of gametes, and genetic assessment. Information systems require the development of a database that is accessible by gene bank managers and the public at large. From the database, users should be able to assess the status of the collection, determine how well it represents various populations, and determine if there is material of interest that can be of potential use. Currently the NAGP animal database is undergoing a revision. In developing this new version the national programs from Canada and Brazil have joined the effort. The database can be accessed via the internet at: *www.ars.usda.gov/Main/docs.htm?docid=16979*.

Reproductive biology and cryopreservation play a critical role in collection development. Cryopreservation techniques are needed to preserve gametes and reproductive systems must be well understood so that populations can be reconstituted with the cryopreserved germplasm. Clearly, technologies have to be developed to secure the female genetic complement. This may require the extraction and cryopreservation of primordial germ cells, or the harvesting, cryopreservation and transplanting of gonadal tissue to accomplish complete genetic security.

Understanding the genetic diversity captured in the collection and how it compares to *in-situ* populations is important for gene bank managers to focus collection efforts and to determine if sufficient genetic resources from specific populations have been collected. It would appear that the utilization of molecular DNA markers such as microsatellites or single nucleotide polymorphisms (SNP) can fill this need as demonstrated by Johnson et al. (2007) and Waldbieser and Wolters (1999).

Germplasm Collection Development

Ex-situ conservation with cryopreserved germplasm is the primary component of the U.S. conservation strategy. The collection and storage of germplasm is a uniquely public sector activity. Furthermore, *ex-situ* collections should be built with several potential uses in mind, including: population regeneration (in worst-case scenarios), maintenance of genetic diversity in populations of interest, providing a source of diversity that industry can use when market conditions change, providing a source of germplasm for the development of new research or industry lines, and providing a source of DNA for research purposes. As cryopreserved collections are built, it becomes evident that there is a need for collections to be subdivided into different categories to meet the variety of potential needs listed above. With the assistance of the NAGP species committees, collection categories, gene bank function and how the various components of the collection can be accessed have been delineated (Table 1).

Table 1. Within-breed components used to define categories, their function and access for the collection of the USDA National Animal Germplasm Program in Ft. Collins, Colorado.

Category	Function of collection	Access to germplasm
Core	Provide sufficient quantities and diversity of germplasm for 150% of breed regeneration*	National, industry, or breed emergency
Evaluation	Sufficient material to evaluate germplasm quality and genetic diversity over time	As needed by NAGP
Working	Germplasm for industry and research utilization for new or experimental line development or DNA studies	Requestor submits a proposal to Species Committee
Restricted	Provides a security backup for private sector germplasm	Permission from germplasm owner

* 150% regeneration is the amount of germplasm necessary to regenerate a breed 1.5 times from cryopreserved material such as semen, embryos, or a combination of both (Blackburn 2004).

Since the initiation of the NAGP, germplasm acquisition across life forms has increased annually at an exponential rate. Currently the collection has more than 10,000 animals in the collection and approximately 500,000 units of germplasm and tissue. As a result of this growth the collection is now the largest national collection of animal germplasm. In addition, at this time, it is the only national collection that contains germplasm from aquatic species.

Collection goals have been established at 2,500 units of germplasm and at least 50 males for finfish species. With these quantities of germplasm, populations can be reconstituted using a back-crossing scheme. In this scheme, the fifth generation of fish will have 97% of the targeted genome. By having 50 males to use in this reconstitution process inbreeding will increase at a rate of 1% per generation which is a target set by FAO (1998). Where needed these goals can be modified so that a specific line within a species can be increased to the targets of 2,500 units and 50 males.

Table 2. Summary of the aquatic species germplasm collection[1] at the USDA National Animal Germplasm Program. The current inventory is database can be viewed at: *www.ars-grin.gov:8080/j2ee/nagppub/jsp/nagp/drilldown2.jsp*

Species	Number of populations	Number of individual animals	Units of germplasm
Freshwater finfish			
Cyprinus carpio	-	1	11
Ictalurus furcatus	-	21	2,247
Ictalurus punctatus	2	52	3,026
Lepomis macrochirus	-	7	174
Morone chrysops	2	21	280
Morone saxatilis	2	52	1,536
Moxostoma robustum	-	28	1,079
Oncorhynchus clarki	7	73	403
Oncorhynchus mykiss	9	365	9,907
Perca flavescens	-	49	506
Polyodon spathula	-	5	552
Scaphirhynchus albus	-	3	72
Xyrauchen texanus	-	13	651
Marine invertebrates			
Acropora palmata	-	4	28
Crassostrea gigas	3	193	6,134
Crassostrea virginica	-	16	1,036
Marine finfish			
Cynoscion nebulosus	-	2	32
Lutjanus campechanus	-	5	143
Lutjanus griseus	-	1	62
Rachycentron canadum	-	1	179
Sciaenops ocellatus	-	5	289
Thunnus atlanticus	-	1	118
Totals for aquatic species	-	729	28,465

[1]As of November, 2010.

The aquatic component of the NAGP collection is approximately 5% of the total number of samples (Table 2). Significant quantities of germplasm have been acquired for channel catfish *Ictalurus punctatus*, rainbow trout, and Pacific oysters *Crassostrea gigas*. Of particular interest are the rainbow trout subpopulations. Contained in this collection are wild populations used by the Colorado Division of Wildlife in their hatchery system, trout from an Idaho hatchery, a commercial line and two selection lines (diploid and tetraploid) that are under development by the USDA National Center for Cool and Cold Water Aquaculture Leetown, West Virginia. The NAGP also has samples from the Hofer line as a backup for the Colorado Division of Wildlife's breeding program. The Hofer line was formed in Germany and re-imported to the U.S. because it confers resistance to whirling disease. While the genetic diversity of the rainbow trout collection has not been quantified, the geographic distribution of the populations collected would suggest a wide sampling of diversity has been captured.

Significant collection development has occurred for aquatic species but substantial efforts are still required to capture and preserve the genetic diversity. In many instances such collections will be dependent upon the development of appropriate cryopreservation procedures as mentioned above.

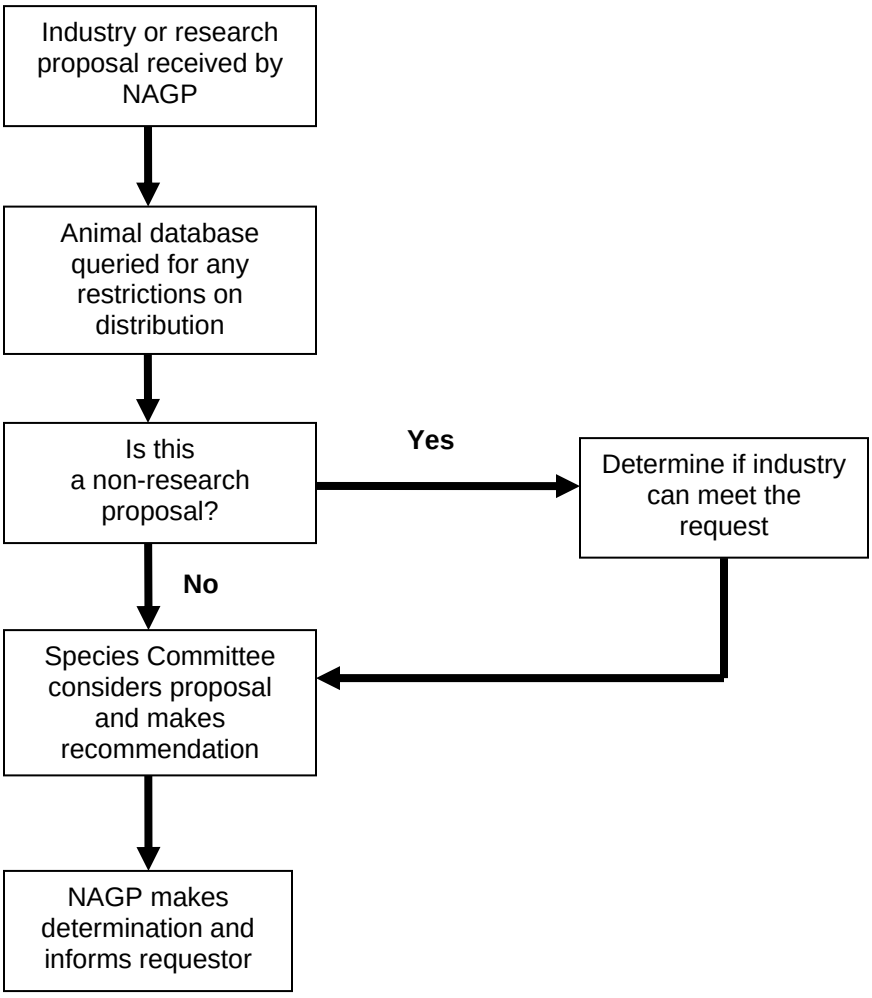

Figure 1. Process for reviewing industry or research requests for animal germplasm or tissue (from Blackburn 2008).

Using the Collection

While the aquatic germplasm collections are meant to be a secure reserve it is also meant to be used by the public and private sector. Conditions have been established for accessing various components of the collection (Table 1). In order to access material in the collection potential users must make a formal request which will undergo a review process (Figure 1). As with all germplasm held by the U. S. government, it is freely available to the requestor if sufficient quantities are available for the requested use. To date, the aquatic collection has been used to cross spring- and fall-spawning research lines of rainbow trout. With other animals the collection has been used in various DNA studies, reconstitution of research populations, and

introduction of genetic variation into a rare breed of cattle. It is anticipated that over time the aquatic species collections will be used in the same manner.

Conclusions

Due to the collection efforts to date the level of genetic resource protection of the NAGP has increased substantially. However, significant efforts are needed to complete the collection process, understand the diversity captured, and provide information about the collection to potential users. Key areas for future program development include: 1) continued collection development for targeted species; 2) development of baseline genetic diversity levels for species of interest; 3) improved cryopreservation protocols across species and tissues types, and 4) increasing the capacity of the information system to facilitate collection utilization, a better understanding within-species genetic diversity, and providing users with a robust set of tools to assist in managing animal genetic resources.

Acknowledgments

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New Chapter

Germplasm Repository Programs in the United States Fish and Wildlife Service

William R. Wayman

Mission of the Agency

The mission of the United States Fish and Wildlife Service (USFWS) is working with others to conserve protect and enhance fish, wildlife, and plants and their habitats for the continuing benefit of the American people. The Fisheries Program of the USFWS has played a vital role in conserving and managing fish and other aquatic resources since 1871 (previously named the Commission of Fish and Fisheries). Today, the Fisheries Program is a crucial partner with states, tribes, other governments, private organizations, public institutions, and interested citizens in a larger effort to conserve these important resources. The Fisheries Program has an important role in restoration and recovery of listed and unlisted species, as well as ecosystems, and maintenance of self-sustaining populations in a healthy condition. The Fisheries Program also conducts numerous activities to fulfill Federal responsibilities associated with mitigating for the adverse environmental effects of water development projects constructed and operated by federal agencies. The Fisheries Program consists of 70 National Fish Hatcheries, 65 Fish and Wildlife Conservation Offices, 9 Fish Health Centers, 7 Fish Technology Centers, and 1 Historic National Fish Hatchery.

Conservation Status of Freshwater Aquatic Species in the United States

Currently in the United States, there are 1371 species listed as threatened or endangered under the Endangered Species Act. Of the 578 animal species listed, at least 250 (43%) are aquatic species (www.fws.gov/endangered/species/us-species.html). One-third of the Nation's freshwater fish species are threatened or endangered, 72% of freshwater mussels are imperiled, and the number of threatened and endangered species has tripled in the last 20 yr (USFWS 2004). Many unlisted species are also in decline or at risk from the same threats that affect listed species. Working with partners, the USFWS uses a range of conservation tools to recover endangered and threatened species. One such tool is cryopreservation.

Cryopreservation is the freezing, storage and thawing of viable biological material. Cryopreservation of sperm, eggs, or embryos can aid in the conservation of these resources by: 1) conserving valuable genetic diversity from declining populations; 2) alleviating the problems of non-coincident breeding seasons or shortages of males by allowing spawning of broodstock when females are ready; 3) facilitating production of appropriate crosses by providing a large pool of available males; 4) reducing the need to handle and house large numbers of male broodstock, which allows greater resources to be devoted to females and offspring, and 5) enabling the movement of genes from wild populations into hatchery broodstocks or breeding programs without the need to remove the animal from the wild.

Wayman, W. 2011. Germplasm Repository Programs in the United States Fish and Wildlife Service. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 780-782.

USFWS Facilities Involved in Repository Development

Research into the cryopreservation of gametes from aquatic species has been conducted by the Service since the early 1990's. This work included cryopreservation of sperm from Apache trout *Oncorhynchus apache* (David et al. 2000), razorback sucker *Xyrauchen texanus* (e.g., Tiersch et al. 1998), Colorado pikeminnow *Ptychocheilus lucius* (e.g., Tiersch et al. 2004), and the humpback chub *Gila cypha* (Gorman 2000).

The Warm Springs Fish Technology Center (FTC) in Warm Springs, Georgia, began research into cryopreservation of fish sperm in 1999. Over the past 11 yr, the FTC has developed a program focused on cryopreservation of gametes from aquatic species and cryopreservation protocols for more than 20 species of fish (e.g., Horváth et al. 2005). This has included construction of facilities and specialized capabilities (Figure 1) to allow research to be conducted directly in the field (e.g., at a remote riverside location) or at cooperating hatcheries while maintaining a disinfection protocol to minimize or eliminate the spread of parasites or disease.

Figure 1. Portable egg incubation systems. Left) a prototype system that could be transported to a riverside location for on-site fertilization trials to evaluate cryopreservation treatments. Right) a self-contained, temperature-controlled, recirculating system built on a trailer that can be disinfected and transported for cryopreservation experiments.

The FTC also maintains a repository of cryopreserved sperm for several species. For example, the repository contains sperm from more than 120 males of the endangered pallid sturgeon *Scaphirhynchus albus*. This repository has been used in restoration efforts for this species by providing additional crosses during spawning efforts, which helps to maintain the genetic diversity of the extant population. Other recent research projects have focused on the development of cryopreservation techniques for sperm of salamanders, and sperm and glochidia from freshwater mussels.

The USFWS has also developed a Memorandum of Agreement with the Unites States Department of Agriculture for storage of cryopreserved material at the National Animal Germplasm Program in Ft. Collins, Colorado. This agreement allows storage of valuable germplasm in a state-of-the-art storage facility while maintaining sole ownership of the samples, providing a safe back-up site to protect these samples.

Acknowledgments

These efforts have been funded in part by the U.S. Fish and Wildlife Service, Louisiana State University Agricultural Center, and the U.S. Army Corps of Engineers. References to trade names or proprietary products does not imply endorsement by the U.S. Government. Links to external servers do not imply any official U.S. Fish and Wildlife Service endorsement of the opinions or ideas expressed therein, or guarantee the validity of the information provided. Links to commercial sites are in no way an endorsement of any vendor's products or services.

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Original Chapter: Revised by Author

The Application of Cryopreservation in Fish Genetic Conservation in North and South America

Brian Harvey

Spotlight on Freshwater Biodiversity

Until very recently, loss of freshwater biodiversity has largely been ignored in favor of mammalian and avian species. It is only now that the conservation community has begun to awaken to the richness and vulnerability of aquatic biodiversity and the emphasis is still on marine fish species -- which make up only a little more than half the total number of fish species (Harvey 2002).

Since about 1992, fisheries scientists have recognized the urgent need to preserve the naturally occurring genetic diversity in wild fish stocks. With the signing of the Convention on Biological Diversity in 1992, nations now have a responsibility, and a mechanism, for putting genetic conservation into practice. Unfortunately, fish genetic conservation lags far behind the much better established conservation of plant genetic resources and the drastic decline of many fish stocks means that we have little time to act.

The high risk of extinction of many genetically distinct stocks of Pacific and Atlantic salmon in Canada is reflected in increasing numbers of listings under the Canadian Species at Risk Act (SARA) and the Endangered Species Act in the U.S. Fisheries restrictions and closures have failed to bring back many declining Pacific and Atlantic salmon stocks and the effect of climate change on ocean survival is an alarming complication. In South America, a dozen migratory species with high economic value and potential for culture face threats that include pollution, habitat loss and dam construction; many stocks have dwindled to the point where irretrievable loss of genetic diversity is plainly occurring (Carolsfeld et al. 2004, Godinho 1998).

Genetic Conservation and Gene Banking

Genetic conservation is a fundamental goal of the Convention on Biological Diversity, and the importance of the genetic diversity contained in wild fish stocks has received increasing attention (Greer and Harvey 2004). Fish gene banks have operated for a decade in Norway (where they were necessary to halt genetic erosion due to acid rain, parasite infestation and genetic pollution), and have more recently been implemented in a number of other countries including Finland, Iceland, India, Russia, Philippines, Canada, the United States, Brazil, Colombia and Venezuela. The largest collections are in Norway and Russia. Precipitous declines in some wild stocks have prompted the recent creation of small, targeted collections; for example, in 1998 the Ministry of Fisheries in British Columbia established a gene bank for several endangered steelhead salmon stocks, and the Canadian Department of Fisheries and Oceans (DFO) maintains collections of selected Pacific salmon stocks.

The term “gene bank” has been broadly defined as “any collection of genetic material kept to ensure the future availability of that material for conservation, study or production” (Pullin et al. 1998). An *ex situ* gene bank of cryopreserved sperm, although not an end in itself, can be a convenient repository of genetic material that might otherwise disappear before habitat

Harvey, B. , 2011. The Application of Cryopreservation in Fish Genetic Conservation in North and South America. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 783-788.

rehabilitation or management measures succeed. Such gene banks are also efficient complements to captive broodstock programs or "living gene banks," by permitting a significant increase in effective breeding number (Harvey 1987). In general, gene banks permit the rebuilding of depleted populations using the original genetic makeup of the stock; in other words, gene banks ensure that restocking is based on the original wild stock, not on an imported hatchery stock. In Norway, where fish gene banking has gone on since 1986, frozen sperm is being used successfully to rebuild damaged runs; in Canada, cryopreserved sperm from Cultus Lake sockeye salmon play a role in the captive broodstock program for that stock.

The technical justification for gene banking lies in its power to dramatically increase the effective population size when broodstock are in short supply. Gene banking is particularly recommended in cases where the breeding technology for repopulating an area is still being worked out, but surviving adults in the wild have reached sufficiently low numbers to cause an irreversible genetic bottleneck (Harvey 1990). This is the case with many South American species: wild stocks are vanishing before the means to rebuild them can be implemented.

World Fisheries Trust's Experience in Genetic Conservation

World Fisheries Trust (WFT) played an active role in cryopreserving fish sperm in the field between 1990 and 2006. Most of the projects focused on collection and preservation of sperm from wild stocks, although WFT also provided cryopreservation and sample management services to the Canadian aquaculture industry, primarily for safeguarding broodstock lines. In Canada, the bulk of WFT's work was on salmonids (five species) and black cod *Anopoploma fimbria*; in South America WFT developed field methods for cryopreserving sperm from a number of migratory fish species of the genera *Salminus*, *Leporinus*, *Piaractus*, *Brycon*, *Pseudoplatystoma* and *Prochilodus* (Carolsfeld et al. 2003)

Efforts in the field have also led to an advisory role in the development of policy for fish genetic conservation, in which capacity WFT continues to collaborate with international agencies, provincial and national governments and First Nations (Figure 1).

Photogragh by B. Harvey

Figure 1. Carrier-Sekani First Nation trainees gene banking Endako River chinook, 1996.

Technology and Methods

World Fisheries Trust collected and cryopreserved fish sperm exclusively in the field. In most cases this involved capture of wild fish, usually relying on local methods and expertise. Permits to capture adult fish and collect milt were obtained from the appropriate agency, and WFT traveled to the spawning site with a field kit containing straws, cryoprotectants, record-keeping materials and cryogenic containers. WFT used commercial dry shippers in a variety of sizes for cryopreservation since 1990.

Donor fish were captured by nets or weirs and milt obtained without anesthesia. Adults were usually released within a few min of capture. Milt was frozen immediately without estimation of sperm motility. We used custom-made numbered 10-mL straws for salmonids and other species that provide large milt volumes; milt from most South American species and tilapias was cryopreserved in 0.5-mL plastic straws manufactured for bull semen.

All field collection data were recorded in SpermSaver, a software program developed by WFT that manages data for each accession and tracks individual samples from the field through storage to eventual use. SpermSaver produces a detailed trip report for each collection, as well as accessions reports for each stock, and is searchable to genus, species, location, straw number, and other fields.

Salmon Gene Banking in Canada

Canada has anadromous salmon populations on both coasts, most of which spawn, die and spend at least half their lives in fresh water. The degree of genetic diversity contained in the many thousands of distinct reproductive populations or stocks makes them an extraordinary example of freshwater biodiversity in their own right, and presents a great challenge in conservation and management of that biodiversity. The depletion and eventual demise of the single-species Canadian Atlantic salmon fishery happened yr before the world's attention had swung to fisheries with the last commercial opening for Atlantic salmon *Salmo salar* in Canada in 1986. But Pacific salmon, of which Canada's six species comprise at least 5,000 genetically distinct stocks, are front and center in the country's collective mind at this time.

Resources like the Pacific salmon fishery remain stable and productive precisely because of their overall genetic diversity. When too many stocks are lost, the variety they represented is edited from the overall gene pool, and the resource becomes highly vulnerable to external stresses like climate change, disease and interception in mixed-stock fisheries (Cloud and Thorgaard 1993).

Salmon gene banking in Canada was led by WFT, in partnership with government agencies and First Nations. Since about 1991, WFT collected and cryopreserved more than 4,500 individual samples from 43 separate stocks of sockeye salmon *Oncorhynchus nerka,* chinook salmon *O. tshawytscha*, coho salmon *O. kisutch,* steelhead *O. mykiss* and Atlantic salmon. World Fisheries Trust did not own the genetic material and did not make decisions about which stocks merited preservation.

In 1992, WFT responded to a request from the Shuswap First Nation for technical assistance in preserving dwindling genetic variability in several species and stocks in southwestern British Columbia. World Fisheries Trust trained workers from this and several

other aboriginal groups, including the Carrier-Sekani First Nation, enabling them to pursue their own gene banking programs.

The Canadian Department of Fisheries and Oceans, the agency responsible for management of most of Canada's anadromous fish species, enlisted WFT's technical assistance in a 2-yr pilot gene banking program prompted by concerns for certain Fraser River stocks of sockeye salmon. World Fisheries Trust collected and cryopreserved 2,500 samples from 750 fish representing 15 stocks in 1995 and 1996. In 1998, conservation concerns for certain British Columbia coho salmon stocks prompted the Department of Fisheries and Oceans to begin its own gene banking program, with technical training and assistance from WFT. To date, that program remains small and targeted on stocks of special concern such as Cultus Lake sockeye.

Since 1993, WFT has provided technical assistance to British Columbia salmon farms interested in preserving broodstock lines. This work usually involves travel to the farm site where ripe donors are provided.

In 2005, WFT transferred responsibility for record keeping and storage of its cryopreserved collections to its clients (owners of the genetic material), while retaining an advisory role.

Gene Banking South American Migratory Species

Canada's freshwater fish biodiversity pales in comparison to that of tropical countries. While it is indeed alarming to learn that 28% of North American freshwater fish species are threatened, endangered or of special concern, comparing the total 177 fish species native to Canadian inland waters with the 2,000 to 3,000 species in the Amazon River alone makes it immediately obvious that diversity is a relative term.

Of the eight major freshwater basins in South America (Magdalena, Orinoco, Amazon, Tocantins, São Francisco, Paraguay, Parana, Uruguay), all but the Magdalena and Orinoco are in Brazil. These colossal waterways and their many tributaries are home to a huge variety of large migratory species that have high economic value in local and national economies but are poorly known outside the South American continent. Many of them undertake prodigious upriver spawning migrations that rival or exceed the better-known journeys of Pacific salmon -- some species travel distances of up to 1,500 km, and most travel more than 200 km every yr to spawn (Carolsfeld et al. 2004).

While some of the native South American species have been successfully cultivated on a commercial scale (and others continue to be added to the list), most of the wild stocks have been steadily declining in abundance and diversity for decades. As is the case with salmon stocks in North America, the causes are complex and include overfishing, loss of habitat, pollution and the construction of dams that block spawning migration routes.

In Brazil, WFT began a formal genetic conservation training program in 1997, following up on several yr field trials with a variety of migratory species in Venezuela, Colombia and Brazil. The project in Brazil was a partnership with Brazilian universities, hydroelectric companies, government and non-government agencies, and equipped biologists from these institutions with the tools for collecting and using genetic material from migratory species. World Fisheries Trust's activities in Brazil were aimed at facilitating broodstock programs for restocking, broodstock programs for culture of native species, and "insurance" gene banking. The technology used was essentially the same as for salmonids, with minor changes in cryoprotectants for some species. Fertilization and incubation protocols were of course different,

and for many species the volume of milt was considerably less than for salmonids (Carolsfeld et al. 2003).

Conclusions

As other chapters in this volume attest, cryopreservation of fish sperm is well established and a variety of techniques provide excellent results. In WFT's experience, modification of our basic field method extended the utility of sperm cryopreservation to a wide variety of warmwater and coldwater species on two continents. Cost and efficacy were not problems -- trainees on both continents have adopted the methods, and even when performed commercially, the fieldwork is no costlier than any other kind of fisheries work.

However, gene banking, especially the collection and cryopreservation of sperm from wild stocks, is often seen as pulling attention and funds away from other methods of conservation, especially habitat restoration. As a "technical fix", gene banking is understandably unpalatable to many, and is pointless unless integrated with all the other measures taken to protect genetic diversity – a requirement that obliges any gene banking proponent to sit down with agencies, aboriginal groups and stewardship organizations *before* any field work is done. In addition, the ability to store fish genetic material for later use usually prompts consideration of ownership issues that local, regional and national governments may not previously have addressed (Greer and Harvey 2004). Gene banking serves conservation, but conservation is a "crisis discipline" where policies are often poorly defined (although the recent Canadian Wild Salmon Policy is a welcome exception). With this in mind, organizations contemplating gene banking of any wild genetic material should at the very least be familiar with the provisions of the Convention on Biological Diversity and should certainly be prepared to cooperate with local agencies to ensure that they are facilitating, rather than inhibiting, the equitable sharing of any benefits derived from those resources, and that their efforts are compatible with more traditional forms of conservation.

Acknowledgments

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The Northwest Salmonid Germplasm Repository

Joseph G. Cloud, Robyn Armstrong, Paul A. Wheeler, Paul A. Kucera and Gary H. Thorgaard

Introduction

Pacific salmon in the Snake River basin exist as a number of spawning aggregates in six major river subbasins, located 500 to 800 miles from the ocean above eight major hydroelectric facilities. Conservation of the Pacific salmon species and steelhead rainbow trout *Oncorhynchus mykiss* in a three-state area with multiple governmental jurisdictions is extremely complex (Figure 1). Species conservation through population protection and monitoring has not been successful to date. These subpopulations, and the genetic diversity contained within them, have been threatened with localized extirpation (Nehlsen et al. 1991, Kucera 1998). As a result, Pacific salmon were officially listed as threatened under the Endangered Species Act in 1992, and steelhead were listed in 1997. Given that the genetic diversity within existing spawning aggregates is not replaceable and should be conserved to protect present and future opportunities, including the evolutionary process in salmon (National Research Council 1996), a salmonid germplasm repository was established.

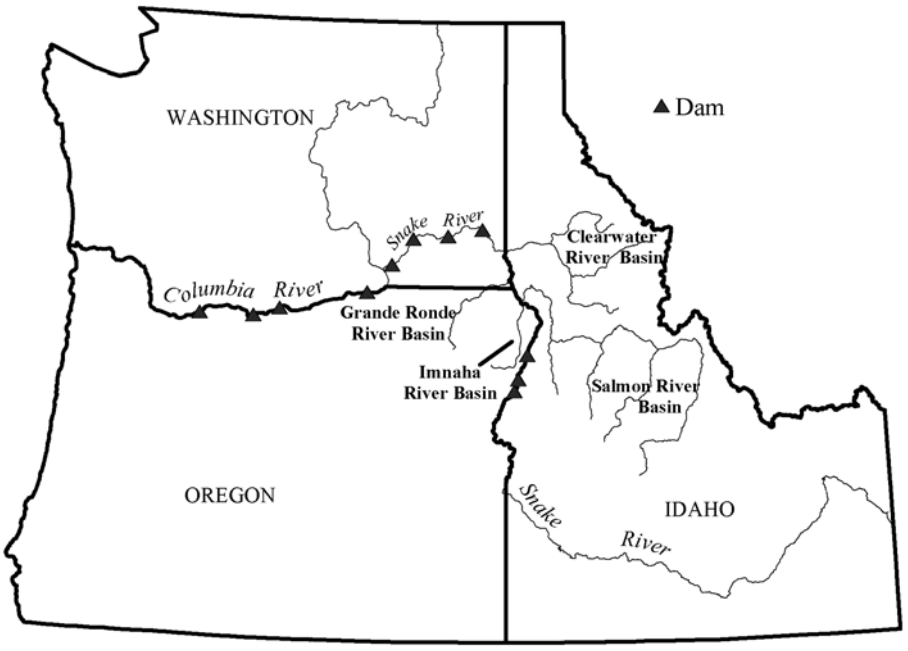

Figure 1. Location of dams in the spawning areas of Pacific salmon and steelhead rainbow trout.

Cloud, J. G., Robyn A., P.A. Wheeler, P. A. Kucera and G. H. Thorgaard, 2011. The Northwest Salmonid Germplasm Repository. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 789-793.

Sample Collections

Our efforts of genetic resource conservation through the application of cryogenic technology for selected populations of chinook salmon *Oncorhynchus tshawytscha* began in 1992. The initial collections were made without knowledge of the genetic variability of the populations or the gene flow among stocks. Because it was difficult to precisely identify the populations from which to sample and the proper number individuals that needed to be sampled per spawning aggregate to preserve the genetic diversity, the collections were based on a geographic river sub-basin approach that was assumed to reflect potential metapopulation structure with reproductive adults returning from the ocean at multiple ages, usually at age groups III through V. Therefore, sampling was structured for a minimum of 5 consecutive yr to ensure that unrelated individuals were represented in the germplasm collection.

The formation and maintenance of the germplasm repository has been a cooperative effort of three groups: the Nez Perce Tribe, the University of Idaho and Washington State University. Each of the groups of this team provides a needed function. Personnel of the Department of Fisheries Resources Management of the Nez Perce Tribe have the responsibility of identifying the spawning aggregates, and collecting and shipping the milt to the two universities. Under ideal conditions, milt from each male is sent to each university. At each university, the samples are frozen and stored independently. In fact, to increase the level of security for the samples, cryopreserved sperm from each male is stored in two different locations at each university. Therefore the genetics of each male is located in four different liquid nitrogen (LN_2) tanks; exceptions to this rule occur as a result of small milt samples.

A summary of the germplasm repository is presented in Table 1 (this Table does not include the chinook salmon collected in the fall of 1999); this information is an extension of the data previously described by Thorgaard et al. (1998). Because of the absence of funding during the formative period, the numbers of collections during the initial yr were modest. However, in 1998, sperm were collected, frozen and stored from 321 chinook salmon males. In total, the repository presently contains sperm from a total of 512 chinook salmon males.

The addition of steelhead to the germplasm repository was initiated in the spring of 1998 with the collection and storage of sperm from 25 males. This effort with steelhead was increased in the spring of 1999 with milt from an additional 167 males. The total straw count to date is 17,920 (Table 1); this total count represents both 0.5-mL and 5-mL straws.

Cryobiological Considerations

In the development of the sperm bank, a number of decisions have been made and a number of procedures have been adopted. Some of the decisions or protocols were based on information available while others were based on our experience.

The Time Period Between Collection and Cryopreservation

The first decision to be made under this category was whether to have a portable laboratory and freeze the milt at streamside or at hatcheries. Although our team periodically revisits this decision, it has been decided that the university laboratory provides the best environment as defined by quality control and the production of a consistent product. Although there does not appear to be a significant difference in the fertilizing ability of sperm frozen on the d of collection as compared to samples held for 24 hr at 4 °C prior to freezing (Cloud, this

volume), the sperm are frozen on the d of collection. Because some of the collection sites are in remote areas with primitive roads, the milt is routinely transported by air to the Moscow–Pullman area using a local charter service.

Table 1. Total Number of male chinook salmon and steelhead that provided sperm in spring and summer collections from locations in the Snake River basin from 1992 to 1999. The cryopreserved samples are stored in 0.5-mL and 5-mL straws.

Collection Site	Species	Number of Males	Number of Straws
1. Lostine River	Chinook salmon	13	669
2. Little Sheep Creek	Steelhead	55	671
3. Imnaha River	Chinook salmon	152	4,344
4. Clearwater River	Steelhead	61	1,171
5. Rapid River	Chinook salmon	98	2,389
6. Salmon River (South Fork)	Chinook salmon	109	3,087
7. Lake Creek	Chinook salmon	10	298
8. Big Creek	Chinook salmon	24	787
9. Capehorn Creek	Chinook salmon	8	263
10. Marsh Creek	Chinook salmon	6	179
11. Snake River	Steelhead	76	1,256
12. Salmon River (Upper/Sawtooth)	Chinook salmon	92	2,806
Totals		**704**	**17,920**

Methods of Handling Milt from Collection to Cryopreservation

One of the more important considerations in the development of a successful sperm banking program is to educate the individuals involved about proper handling and packing the milt for shipping. Everyone needs to understand that sperm are live cells that respire, and that the temperature and pH of the system are important.

Milt was collected from male chinook salmon captured by hand or with nets from streams or from hatcheries. The milt was stored in plastic bags filled with air from a pump and kept in a cooler at 4 °C. In previous yr the bags containing the milt were gassed with pure oxygen. As a result of the information provided by Bencic et al. (1999), the protocol was changed. This change negated the need to pack oxygen cylinders into remote locations; the main caveat to this practice is that the bags cannot be filled by blowing into them because these is sufficient carbon dioxide in exhaled air to significantly reduce the pH of the seminal plasma and the fertility of the sperm. Two things need to be kept in mind at this point. Firstly, more is not always better; filling the bags too full prevents adequate diffusion of the gases and can result in a reduction in fertility. Secondly, the temperature of the ice is the temperature of the freezer from which it came. Transporting the sperm in a cooler that is cold enough to freeze the milt during transport is not recommended.

Estimating Sperm Motility

Motility evaluation is an important component of a cryopreservation program in order to cull poor quality semen samples prior to freezing, and to estimate the fertility of the stored sperm. The rationale for culling poor quality samples is that the cryopreservation process is costly in terms of personnel, time and materials, that space in storage tanks is finite and that the eggs to be fertilized by the cryopreserved sperm may be quite valuable. As a standard practice, all milt samples are evaluated just prior to freezing. Except for milt from wild fish, samples with less than 20% motility are discarded.

The Amount of Sperm from Each Male to be Stored

One of the decisions that needed to be made was the quantity of sperm that would be cryopreserved for any one male. This decision was required because there is a finite amount of space in the storage tanks and the belief that the genetic representation of a male should not be dictated by the amount of milt available. As a result, each male is limited to a maximum of 22.5 mL of milt. The milt is diluted 1:3 with a solution (300 mM glucose, 10% dimethyl sulfoxide, 13.3% egg yolk). At each university, the milt is immediately packaged in twenty 0.5-mL straws; additional milt is packaged in 5-mL straws (not to exceed five additional 5-mL straws for any male). At a maximum, the genetics of each male is contained on two canes (each containing ten 0.5-mL straws) and in five 5-mL straws at each university.

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Annotated Bibliography of Developments in the Last Decade

Since the first review on salmonid sperm was published in 1980, there have been more than 400 publications addressing this topic. More attention is now paid to specific salmonid species and populations from a variety of watershed regions.

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New Chapter

Cryopreservation of Germplasm for Effective Management of Animal Genetic Resources

William Rall, Franziska Grieder and Michael C. Chang

Over the past century, aquatic animal species have demonstrated their utility as powerful models for studying human development, behavior, genetics and disease. Zebrafish *Danio rerio*, medaka *Oryzias latipes*, swordtails and platyfish of the genus *Xiphophorus*, and the African clawed frog *Xenopus laevis* are increasingly valuable to biomedical research as new genetic and genomic technologies and new information emerge from DNA sequencing efforts. Because aquatic species share a surprising number of physiological processes with humans, they can provide critical clues to the biological mechanisms that underlie human health and disease. Aquatic models, particularly zebrafish, are valuable for studies of embryonic development because their eggs are transparent and the developmental processes can be easily observed. In recent years, the National Institutes of Health, National Center for Research Resources (NCRR), Division of Comparative Medicine has extended its long-standing support of these research models by funding efforts to explore and stock promising new aquatic models and to expand existing ones. That support has led to the emergence of a new generation of genomic tools and has revolutionized the way in which developmental and regulatory processes are studied and has enhanced the research value and versatility of a number of these aquatic models. As in other biomedical communities, researchers utilizing aquatic models are now able to produce transgenic, knockout, and mutant lines for the study of human diseases. For example, large- and small-scale genetic screens have led to the isolation of thousands of unique zebrafish strains, and technologies such as TILLING (targeting induced local lesions in genomes) and transgenesis, will continue to add to the number of strains. However, the requirements to maintain strains as live animals can overwhelm the optimal operation of NCRR-supported resource centers.

The NCRR-supported Zebrafish Resource Center in Eugene, Oregon maintains nearly 40% of lines as live populations and continues to acquire hundreds of new lines annually from investigators and institutions conducting small- and large-scale genetic screens. In rodents, cryopreservation of gametes, embryos, and tissues have reduced the cost and demand for animal housing space, as well as reduced the risks of loss by pathogens, and therefore reduced the necessity for importation quarantines. In contrast, the technological advances in cryopreserving fish sperm are suboptimal or non-existent in terms of functional survival and reproducibility and there is no method to successfully freeze embryos. To address this disparity, and to assess the current state of the cryopreservation of a number of other high priority laboratory research species (rodents, rhesus monkeys, and swine), the NCRR Division of Comparative Medicine convened a workshop in 2007 entitled *Achieving High Throughput Repositories for Biomedical Germplasm Preservation*. A major recommendation from that meeting encouraged more research and development of technologies for germplasm collection, evaluation, processing, and cryopreservation to shorten and refine the processes to meet the research community needs for high-quality animal germplasm preservation. The workshop identified the obstacles, opportunities, and priorities that may address the needs for improved cryopreservation methods for each of the critical model species needed for translational

Rall, W., F. Grieder, and M. Chang. 2011. Cryopreservation of Germplasm for Effective Management of Animal Genetic Resources. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch. and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 794-795.

research. The final report including recommendations from the workshop can be found at: *www.esi-bethesda.com/ncrrworkshops/Biomedical/index.aspx*.

These efforts are consistent with the overall goal of NCRR to develop and provide access to critical biomedical models through specialized animal research facilities for clinical and translational research. The facilities supported by the NCRR Division of Comparative Medicine include the Mutant Mouse Regional Resource Centers, the Knockout Mouse Project Repository, the National Resource for Zebrafish, the National Primate Research Centers, and the National Swine Research and Resource Center. A common approach among several of these facilities is cryopreservation of germplasm as the primary mode of storing and disseminating the many genetically altered animals, and development and use of assisted reproductive technologies in addressing infertility of animal models. The application of these technologies have revolutionized the control of reproduction as a result of developments such as artificial insemination, *in vitro* fertilization, cryopreservation of gametes and embryos, embryo transfer, hormonal treatments, intracytoplasmic sperm injection, testicular sperm extraction, and somatic cell nuclear transfer. In many cases, these technologies improve reproduction to the extent that additional germplasm that otherwise would not be available can be collected for establishing banks of cryopreserved gametes and embryos.

The NCRR also supports research that complements cryopreservation projects, such as tools to reduce variability of the quality of sperm, oocytes, embryos, and embryonic stem cells during collection and processing, re-derivation of living young with the same homozygosity as the original animals, methods to screen cryopreserved samples to diagnose and eliminate selected diseases of laboratory animals, and devices and processes for thawing, ultra-rapid cooling, recovery of germplasm, removal of cryoprotective agents, and tests for viability.

A significant emphasis of the NCRR Division of Comparative Medicine is to support research for cryopreservation of germplasm for animal models where survival of cryopreserved sperm and embryos are poor or non-existent, and to encourage the development of high-throughput and scalable technologies for germplasm collection, evaluation, processing, and cryopreservation. To help address this area of research we have sponsored several specific Funding Opportunity Announcements (FOA) requesting applications to address the need for innovative methods and tools to establish cryopreservation methods for targeted species including mouse, rat, rhesus monkey, and zebrafish (NCRR Division of Comparative Medicine FOAs: *ncrr.nih.gov/comparative_medicine/funding.asp*. One example of such a funding opportunity is the ongoing announcement PA-10-138, Development of Animal Models and Related Biological Materials for Research *(grants.nih.gov/grants/guide/pa-files/PA-10-138.html).* Applications are accepted to develop innovative methods and robust tools to establish reliable and standardized protocols for preservation and to secure long-term maintenance of sperm, embryos, oocytes, and other forms of germplasm in aquatic models. Such research may include projects leading to the development of new cryopreservation procedures and tools for stock centers and other large-scale platforms to achieve efficient, reliable, reproducible, and cost-effective methods for genetic banking, as well as research leading to the transfer and improvement of existing reproductive technologies originally developed for the mouse (e.g., intracytoplasmic sperm injection and embryo cryopreservation) to aquatic species. These FOAs present a unique opportunity to develop tools necessary to enable repositories to establish banks of animal germplasm for current and future research needs.

New Chapter

Xiphophorus *Fishes: Varieties and Genetic Resources*

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The Variety of *Xiphophorus* Fishes

Xiphophorus are small freshwater fish that are often placed into two groups, platyfish and swordtails (Kazianis and Walter 2002, Kallman and Kazianis 2006). In addition to being commonly found in the aquaria of hobbyists, *Xiphophorus* has a long and storied history of contributions to scientific research. The use *Xiphophorus* in the aquaria trade and in research stems from the extreme variability found among the 26 known species (for an excellent review of the genus *Xiphophorus* see Kallman and Kazainis 2006).

In the wild, *Xiphophorus* fishes are found distributed from northern Mexico about 200 km south of the United States border and extend south into to Central America. Natural habitats include various freshwater drainages supplied by the Sierra Madre Oriental geographic uplift and southward traversing the continental divide ultimately extending into Guatemala, Belize, and Honduras. The geographic isolation of localized regions within this large area is replete with highly variable habitats. From relatively stagnant waters and springs at sea level to fast flowing mountain streams, these environments have given rise to many small and isolated *Xiphophorus* populations (e.g., *X. meyeri, X. couchianus,* and *X. gordoni*). However, there are also species that have extensive and sympatric ranges nearly traversing Mexico (e.g., *X. variatus, X. helleri,* and *X. maculatus*). Thus, one hallmark of *Xiphophorus* is the high degree of morphological variability among species (Figure 1, next page). In addition, all *Xiphophorus* are live-bearers and it is enticing to speculate how this reproductive mode may have played a role in producing the high degree of speciation observed among these fish.

Species Conservation of *Xiphophorus* in the Wild

The locations where *Xiphophorus* are found in Mexico and Central America represent some of the most species-diverse areas in the world. However, as with the rest of the world, these regions are experiencing increased pressure on freshwater resources due to demands of human population growth, urban expansion, and the necessity of industrialization. Of 506 freshwater fish species known to exist in Mexico, 185 are listed as “endangered” and there have been 20 recorded extinctions (Contreras-MacBeath 2005). Of the freshwater fishes, the family Poeciliidae which contain the genus *Xiphophorus*, is among the most exposed to habitat loss (Contreras-Balderas 2005, Contreras-MacBeath 2005). Of 86 species in the family Poeciliidae, 20 species are considered to be stressed or “at risk” with 8 endangered, 9 threatened, 2 under special protection, and one extinct. Of these, 5 species of *Xiphophorus* (Table 1, page after next) are listed as endangered, and at least one of these may be extinct in the wild. Efforts on the part of governments to set up species protection regimens are often focused primarily on more popular or high-profile species such as sea turtles, jaguars, bears, and monkeys. Thus, the impacts of population growth and water management on the sustainability of freshwater fishes remain largely unaddressed. This worldwide problem will reach greater significance as regional water issues become more prominent on political and social agendas. The plight of the *Xiphophorus* species represents one group already showing adverse effects. In addition, the changing

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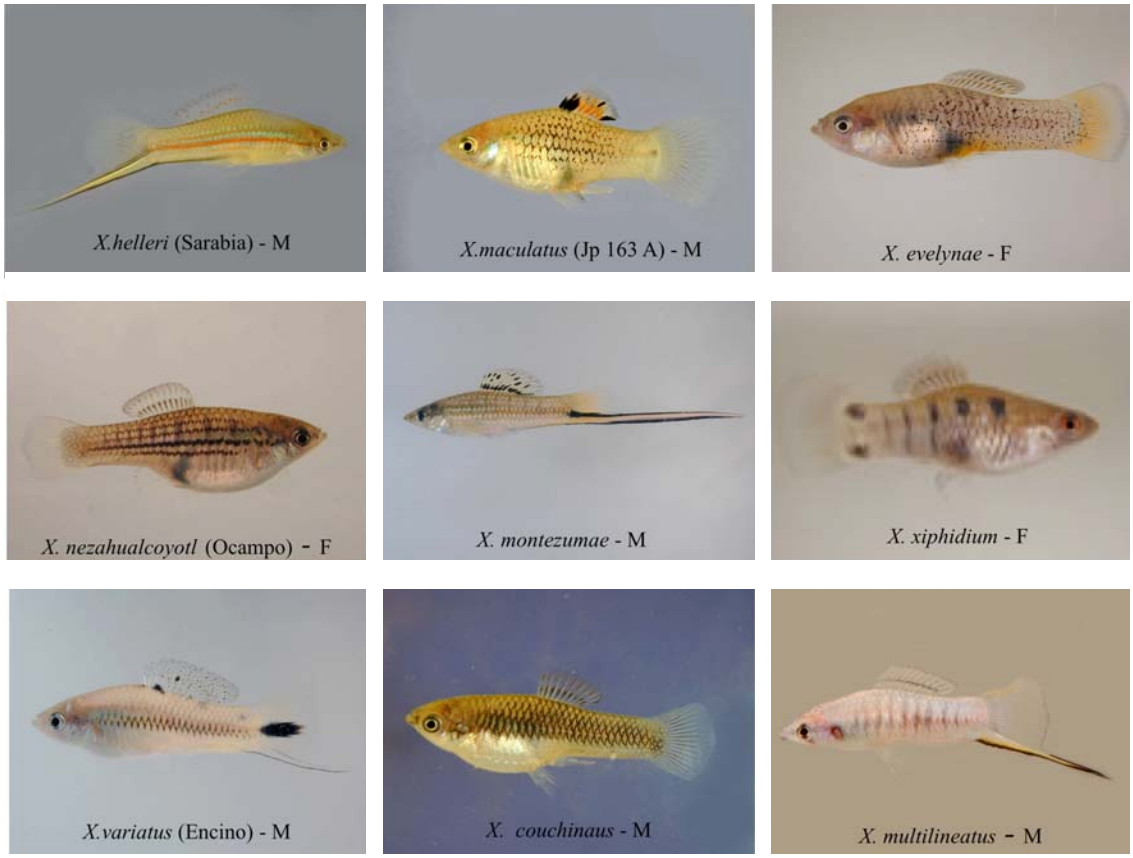

Figure 1. Examples of the morphological variability within the genus *Xiphophorus*. "M" or "F" designates male or female.

environment may serve to further confound species protection mechanisms. A recent 15-model consensus projection of the regional impact of climate change on North America shows the Pacific drainages of northern and central Mexico may be "hot spots" for greater than normal effects in climate modulation (Kerr 2008). The larger responsiveness of these regions to climate change may exacerbate loss of freshwater habitats and hasten requirements for species preservation programs to be put in place.

Xiphophorus as a Research Model

To better understand multigenic or complex traits, the development of appropriate animal models is of considerable importance. Informative and useful animal models should have characteristics that make them amenable to controlled experimentation. Ease of generating large numbers of animals, the availability of inbred stocks and strains, robust genetic and molecular genetic resources allowing determination of gene similarity with other models, and the ability to perform genetic crosses for mechanistic investigations represent just a few characteristics of a good model system. Collectively, *Xiphophorus* fishes possess these and other favorable attributes that serve to promote experimental use of these fish internationally. In contemporary research *Xiphophorus* fishes are actively used in many fields of study, including evolution, behavior, physiology, comparative biochemistry, comparative genomics, sex determination,

Table 1. List of *Xiphophorus* species registered as threatened based on data from the Norma Official Mexicana (NOM 059-ECOL-2001)*. It bears mention that all of these except *X. milleri* are stated to be of "high priority" indicating they have a higher probability of extinction.

Species	Distribution**	Class	Threats**
Xiphophorus couchianus	Springs near Monterrey, Nuevo Leon	Northern Platyfish	Urban expansion of Monterrey
Xiphophorus gordoni	Cuatro Cienegas, Coahuila	Northern Platyfish	Pollution, invasive exotic species
Xiphophorus meyeri	Springs near Rio Salado, Coahuila	Northern Platyfish	Dewatering, pollution
Xiphophorus milleri	Laguna de Catemaco, Veracruz	Northern Platyfish	Pollution, invasive exotic species
Xiphophorus clemenciae	Rio Coatzacoalcos, Verecruz and Oaxaca	Southern Swordtail	Agriculture, colonization

*Contrreras-MacBeath 2005

**Contrreras-Balderas 2005, and Kallman and Kazainis 2006

development, endocrinology, behavioral ecology, toxicology, parasitology and immunology (Table 2). Interest in using the powerful genetics provided by the *Xiphophorus* model system has increased over time. Since the late 1800's over 2,000 peer-reviewed publications appearing in scientific journals have detailed *Xiphophorus* research studies and if one looks at the number of publications per decade, a 60% jump in publication rate is observed between the 1980's and the 1990's.

Table 2. Representative recent areas of interest and citations using *Xiphophorus* fishes as models in diverse scientific disciplines.

Field or Discipline	Citation
Evolution and biogeography	Kallman and Kazianis 2006, Meyer et al. 2006, Wilkins 2004, Meierjohann et al. 2004, Myer 1997
Behavior	Wong and Rosenthal 2006, Basolo and Alcaraz 2003, Earley et al. 2003, Beaugrand and Goulet 2000
Physiology	Yang et al. 2007, 2006, Dong et al. 2006, Pinisetty et al. 2005, Chano et al. 2004
Cryobiology	Huang et al. 2004a, 2004b, Leknes 2004
Comparative biochemistry and genomics	Ju et al. 2005, Walter et al. 2004, Oehlers et al. 2004 David et al. 2004, Heater et al. 2004
Sex determination	Kazianis et al. 2005, Volf and Schartl 2002, Kallman 1983
Development and ageing	Kallman 2005, Royle et al. 2005, Zauner et al. 2003
Endocrinology	Flynn et al. 1999, Breuckmann et al. 1996
Toxicology	Kwak et al. 2001, Gandzyura and Ignatyuk 1998
Parasitology	Pineda-Lopez et al. 2005, Dove 2000, Schmahl et al. 1996
Immunology	Roney et al. 2004, McConnell et al. 1998

Due to their early use in scientific research, *Xiphophorus* represents one of the oldest groups of established inbred genetic strains. At least 21 of the species can be crossed with one another to produce fertile interspecies F_1 hybrid progeny. *Xiphophorus* interspecies crosses present scientists with models of considerable genetic power because the resulting progeny are polymorphic for almost all loci along each chromosome. Thus, specific genes associated with multigenic or complex phenotypes can be identified by linkage analyses performed on cohorts of backcross hybrid progeny.

One drawback of using live-bearing fish such as *Xiphophorus* in scientific investigations is the inability to perform mutational screens. For such studies oviparous aquarium fish models (e.g., medaka and zebrafish) are more appropriate because one may easily acquire the thousands of embryos needed to employ brute force mutational analyses (i.e., N-ethyl-N-nitrosourea style mutagenesis). However, *Xiphophorus* females once inseminated may store sperm for 6-10 months and can produce a brood (5-40 young) about every 30 d. Also, several lines of *Xiphophorus* are highly inbred (over 100 generations, see below) and concomitant mating of siblings can be staged to produce hundreds of genetically uniform progeny having very nearly the same birthdate.

The *Xiphophorus* Backcross Hybrid Experimental Design and Tumor Models

The mating of two *Xiphophorus* species to produce fertile F_1 interspecies hybrids allows one to backcross the F_1 hybrid to one of the parental fish lines and produce cohorts of backcross hybrid (BC_1) progeny. The segregation of parental chromosomes into *Xiphophorus* interspecies BC_1 hybrid progeny leads to a genome carrying, on average, 25% of the chromosomal content of the non-recurrent parent and 75% genomic content from the recurrent parent. For multigenic traits (e.g., tumor development) this mixing of divergent genomes in the same genetic background may lead to disturbances in cellular pathways at the molecular genetic level. Often, these molecular genetic disturbances can be scored by observation of modulated phenotypic expression in those BC_1 hybrids that inherit specific chromosome sets (e.g., in tumor studies, the development of a nodular lesion). Co-segregation of the new phenotype with a defined set of genetic markers, via marker linkage analyses of backcross hybrid cohorts can be used to localize genes involved in expression of the complex trait to specific chromosomal regions. Using this approach, discrete genetic factors that are inherited in a specific manner to predispose the resulting progeny to spontaneous or induced melanoma development have been identified (for reviews see Meierjohann and Schartl 2006, Walter and Kazianis 2001). Data gleaned over the past 80 yr of *Xiphophorus* genetic research have identified genotypes and determined whether a particular BC_1 animal will develop, or has a defined probability to develop, a specific tumor type (i.e., melanoma). However, in principle, the same experimental approach may be employed to dissect the genetics underlying any complex or multigenic trait (i.e., behavioral, or optic or otic sensing) that one can score among *Xiphophorus* parental species.

The power of backcross hybrid genetic analyses is the unbiased nature of the experimental design. This design asks the BC_1 animals to identify phenotypic modifiers by altered expression of the phenotype of interest within the intact animal. Such classical genetic analyses, when coupled with high-density mapping and reverse genetic cloning, have the potential to delineate new genetic interactions and allow isolation of genes having unexpected associations with many complex traits. Although extremely important for mechanistic research study, the rather narrow divergence times inherent to mammalian experimental systems often do not allow for such allelic interplay. However, when such analyses are performed in fish species having genomes that are 65-80 million years diverged, such as *Xiphophorus* interspecies hybrids, complex phenotypes can be dissected readily into genetic components.

Development of the *Xiphophorus* genetic system and its employment in varied scientific fields of inquiry can be traced to pioneering studies by Dr. Myron Gordon and Dr. Kurt Kosswig in the 1920's that showed interspecies BC_1 hybridization of *X. maculatus* and *X. helleri* (backcrossed to *X. helleri*) resulted in progeny that developed melanoma (Gordon 1931, Kosswig 1927, Anders 1967). Results from these early *Xiphophorus* tumor crosses established the presence of what we now term "oncogenes" and were the initial indications that loss of gene function could be associated with tumorigenesis, thus implying the existence of "tumor suppressor genes".

In the years since these pioneering studies genetic control of tumor susceptibility in *Xiphophorus* has been shown to exist in pure strains and in particular interspecies hybrids for a variety of spontaneous neoplasms (due simply to the interspecies cross) and induced neoplasms (requiring interspecies hybridization and ultraviolet B irradiation or chemical inducing treatment). These *Xiphophorus* hybrid crosses include at least 8 different melanoma models and many less well-studied tumors such as neuroblastomas, neurofibromas, fibrosarcomas,

retinoblastomas, and rhabdomyosarcomas (Walter and Kazianis 2001, Kazianis et al. 2001a, 2001b, Schwab et al. 1978a, 1978b, Schwab et al. 1979).

The *Xiphophorus* Genetic Stock Center

Research experiments often require that defined strains of genetically identical animals or plants be used to ensure that results can be repeated in any laboratory and that differences are not due to environmental factors, or to the random genetic variation found in almost any wild population of animals. Among vertebrates, genetically identical animals are usually produced by sustained inbreeding between brothers and sisters: in each generation about one-half of the genetic differences of the parents are lost. Successive inbreeding eventually results in genetic uniformity within a stock. Due to the early melanoma studies by Myron Gordon, one of the oldest and best-defined groups of established inbred genetic strains consists of *Xiphophorus* platyfish and swordtails housed in the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University, San Marcos, TX (see *www.xiphophorus.org*).

As a student at Cornell University in the early 1930's, Gordon nurtured the desire to identify the genetic factors responsible for melanoma development. He began breeding and collecting *Xiphophorus* strains and in 1939 formally established the *Xiphophorus* Genetic Stock Center (XGSC) at the New York Aquarium then located at the Battery in Castle Clinton, Manhattan, NY (for excellent review see Kallman 2001). Over the ensuing years Gordon made many trips to Mexico. In those days he traveled in a Model T ford, loaded with camping equipment and shotguns, carrying letters from various U.S. federal agencies, and explored Mexico looking for new *Xiphophorus* populations. When *Xiphophorus* were found, Gordon would use metal milk canisters to ship fish back home to New York via the railroad. Without maps or many well built roads he expanded the *Xiphophorus* collections in the XGSC. Later, the rapidly growing XGSC collection was moved to the top floor of the American Museum of Natural History in New York City, NY. Upon Gordon's death in 1959, and for the ensuing 30-plus years, the Center was overseen and maintained by Dr. Klaus Kallman, who had trained under Gordon's supervision as a doctoral student. Kallman continued exploratory trips to Mexico and Central America and greatly expanded the XGSC at the New York Aquarium to represent 21 species and over 50 pedigreed fish lines. In 1993, due to Kallman's retirement and the concentration of researchers in Texas using these valuable animals, the XGSC was transferred to Texas State University (TSU) in San Marcos. Several of the original genetic strains of platyfish and swordtails developed by Dr. Gordon in the 1930's are still available at the XGSC; in some cases being the products of more than 100 generations of continuous controlled matings.

The XGSC at TSU occupies about 325 m^2 and maintains ~1,200 20-L and 150 40-L to 200-L aquaria for stock perpetuation. The Center currently houses 25 of the 26 known species of *Xiphophorus* and maintains 63 pedigreed genetic lines. The Center produces custom interspecies hybrid crosses or backcrosses for a variety of research projects on a case-by-case basis. The XGSC staff provides consultation on husbandry and genetic questions and have the capability to establish the genotype of research animals should questions of parentage arise. The XGSC will assist in preparing contracts for fish supply and will provide needed support letters for grant and contract proposals. In addition to fish and fish services, the XGSC has been compiling genetic and genomic tools for other researchers. A few of the current services and databases provided via website at: *www.xiphophrus.org* are briefly detailed below:

Xiphophorus Links, Images, and Bibliography

The XGSC web page includes links to other fish resources centers and *Xiphophorus* research investigators worldwide. In addition, a searchable (via key word, author, citation, etc.) literature bibliography containing >1,800 *Xiphophorus* publications is freely accessible. The web page also contains digital images of many *Xiphophorus* species, images of several backcross hybrid cross diagrams and an educational site providing information about *Xiphophorus* research projects.

Microsatellite Loci Database

This microsatellite database allows a user to search for specific or group records, by name or partial name. It also allows microsatellite files to be searched by specifying a specific backcross hybrid in which a polymorphism was observed or by specifying a parental strain that was screened and yielded polymorphic amplification products (by polymerase chain reaction). The results will yield information such as: the gene map location of the microsatellite locus, the primer sequences used to amply the marker, and a link to a gel image that shows the results of using the microsatellite primer pair with DNA obtained from 9 *Xiphophorus* species and DNA obtained from other model fish species including guppy *Poecilia reticulata* (QUL89-A and PVMSK-D), zebrafish *Danio rerio* (AB ZIRC strain) and medaka *Oryzias latipes* (Carolina Biological Supply).

The Xiphophorus Linkage Map

The XGSC led two large-scale *Xiphophorus* gene map-building studies using linkage analyses of BC_1 hybrids (Kazianis et al. 2004, Walter et al. 2004). Based on the two gene maps derived from these studies, the genome size is estimated to be ~830 megabases (mb), or about 24% of the size of the human genome. The average marker coverage is about one marker per 7.5 cM and the average linkage group size is 90.7 cM ranging from 26.1 (LG 21) to 178.2 (LG 4). Estimates from the mapping yield a physical-to-genetic map ratio (or physical recombination rate) of ~332 kb/cM (or 3.01 cM/mb), which is much higher than that of mouse at ~2,100 kb/cM (0.48 cM/mb) or humans at ~1000 kb/cM (1.0 cM/mb). Thus, *Xiphophorus* clearly have a very high recombination rate in contrast with these well-studied mammals.

An interesting outcome of the two mapping projects was the determination of sex-specific recombination. Comparison of the map length estimates based on a male-only gene map (Kazianis et al. 2004) and the sex-averaged gene map (Walter et al. 2004), suggest that there are no significant differences in sex-specific recombination rates in *Xiphophorus*. Lack of sex-specific recombination differences in *Xiphophorus* hybrids (e.g., the female/male recombination ratio = 1.0) is quite different from the strongly female-biased recombination rates observed in other fish species examined (e.g, female/male recombination ratios in zebrafish of 2.74, and in rainbow trout *Oncorhynchus mykiss* of 3.25) (Sakamoto et al. 2000).

Xiphophorus EST Database

A *Xiphophorus* expressed sequence tag (EST) database, termed XiphoBase, is available. The database houses all current *Xiphophorus* ESTs (with new ESTs added as they are characterized) available through a series of searches. These searches and associated sorting consist of any fields available in the data bank (e.g. GenBank #, EST #, authors, citation, sequence). One can select the fields to be displayed. Searches by sequence or partial sequence

will also be made available. All information regarding these ESTs is available including links to the GenBank file hosting the EST sequence.

Xiphophorus Genome Resources

Over the past decade the XGSC has been working to develop genomic and transcriptomic tools for *Xiphophorus* research. To start this process, bacterial artificial chromosome (BAC) libraries were developed that provide rapid access to large genomic inserts (150 kb or more) for a wide range of research applications. Then bidirectional end sequences from 49,056 *X. maculatus* BAC clones (98,112 total sequences) were produced and placed in a database that contains the accession number, the sequence, the trimmed sequence, and the gene name identified for each sequence by basic load alignment search tool (BLAST) analysis. In addition to BAC end-sequence search data, FPC Software (created at the University of Arizona) is linked to the database located on the www.xiphophorus.org server. The link allows a user to search a selected set of BACs making a contig (a set of overlapping DNA fragments) and yields the fingerprint mapping for each member in the set. In this manner any user can produce individualized BAC Minimal Tiling Path (MTP) contigs using their own criteria for mapping construction.

Very recently the goal of producing functional genomics capabilities was reached by the XGSC spearheading an effort to sequence the *X. maculatus* genome. Concurrently, through collaborative efforts, thirteen separate transcriptomes sequence libraries from distinct developmental stages or select tissues were accumulated to produce a very deep *X. maculatus* reference transcriptome assembly. These resources, once annotated poise *Xiphophorus* as a premier model to study the fundamental mechanics of gene interactions by applying transcriptome sequencing methods (RNA-seq) to various *Xiphophorus* interspecies hybrids.

The *X. maculatus* Jp 163 A genome was sequenced and assembled at the Washington University, Genome Sequencing Center in St. Louis (USA). The *X. maculatus* genome is one of the first to be sequenced and *de novo* assembled using next-generation sequencing platforms (454Ti and Illumina). As of this writing the draft genome assembly (669 Mb) consists of 130,963 large contigs with an N50 contig length of 21 Kb, and 84,533 supercontigs with an N50 length of 1.1 Mb. A large independent set of Illumina GAII data (11-x coverage) will be used to assist in correcting questionable base calls for genome annotation. The draft *X. maculatus* genome assembly will soon be released to the NCBI database and the final genome assembly presented to ENSEMBL for annotation and public posting. The *Xiphophorus* genome assembly is derived from a single female (*X. maculatus* JP 163 A) in the 105th inbred generation. This female was used to start an independent pedigree and her direct descendants have been used in all subsequent transcriptome sequencing and are available from the XGSC.

The Future of the XGSC

It is a great ambition of many scientists to have the *Xiphophorus* genome sequenced and added to the public genome databases. A *Xiphophorus* genome sequence would not only allow scientists to perform comparative analyses that delve into the evolution of live-bearing fishes, but would also serve as a reference genome for new studies aimed at determination of the mechanics behind complex life history traits such as maturation, aging, sexual selection, and behavior. At this juncture there is no better reference genome to post than one that allows scientists to approach novel questions in an evolutionarily distinct group of New World fishes that exhibit high speciation and morphological variability, yet may be crossed to produce fertile interspecies

hybrid progeny. The first steps toward the genomic goal were taken with the construction and complete end sequencing of all clones in the *X. maculatus* BAC library described above. We now have the ability to produce BAC minimal tiling paths through large chromosomal regions and eventually will be able to collate the physical (BAC) and recombination (linkage) maps. These data will assist genome sequence assembly once a large-scale genomic sequencing effort initiates as proposed for the near future. As we enter an era of comparative genomics and comparative genetics, the power of the species-rich *Xiphophorus* model to approach complex scientific problems is very attractive

When the XGSC was transferred to TSU it arrived as a well-documented natural "living library" of diverse strains (i.e., more than 50 lines) and resources (e.g., pedigree data, fixed samples). In the years since it has been in San Marcos, maintaining and expanding this library have been the primary objectives. However, with shrinking resources and shifts in scientific funding priorities away from living resources and basic research models, the capability to maintain all of the volumes of this living library may be lost. When libraries lose volumes they become less valuable and at some point after successive losses they cease to be of value at all.

The XGSC serves as a repository for *Xiphophorus* fishes, those inbred as inherited from Gordon and Kallman, and those newly described, as four recent new species (Kallman et al. 2004, Meyer and Schartl 2002, 2003). At the very least the XGSC reduces collection pressure on natural populations, and if trends continue may harbor the only reference stocks of several species left on Earth (e.g., *X. couchinaus*, *X. gordoni*). The XGSC has maintained support for its operation from various sources, of which the most notable is the National Institutes of Health, National Center for Research Resources that has supported XGSC operation for the past decade. However, even after more than 80 yr of continuous operation, the XGSC (like most resource centers) operates while teetering on the brink of demise. In an effort to preserve some of the genetic heritage of the current 63 lines, efforts have been made to develop cryopreservation protocols for *Xiphophorus* sperm. This process, although laborious, has thus far proven to be successful for three species: *X. helleri* (Huang et al. 2004a), *X. maculatus* (Pinesetty et al. 2005), and *X. couchianus* (Huang et al. 2004b).

We hope to use the advances we have made in cryopreservation to initiate long-term archival sperm storage from species where we have proven cryopreservation is successful. Because no less than 15 fish lines maintained at the XGSC represent rare chromosomes or site location duplicates in *X. maculatus* and *X. helleri*, the preservation of these lines as cryopreserved sperm would substantially decrease the labor at XGSC and allow more hypothesis driven science to be performed. In addition to maintaining chromosomal representation we plan to employ cryopreservation of species that are considered threatened and possibly extinct in wild, such as *X. couchianus*. We also plan to continue to extend protocol development in other species, and establish reliable and standardized approaches to establish a sperm repository for eventual inclusion of all fishes maintained at XGSC. These will serve as a back-up for irreplaceable *Xiphophorus* lines in perpetuity, serve to provide a frozen archive of genotypes at specific intervals in the XGSC timeline, and reduce XGSC labor to maintain lines. The stocks selected for initial long-term cryopreservation are those maintained for a single chromosome variation by continual backcrossing into the *X. maculatus* Jp 163 A line (XSrAr, YSdDr, YSdSr, YSp, JpIrBr, JpYIr, JpYBr), have a different pigment patterns or mutations (e.g., Nigra, Jp30R, *X helleri*-alb, HelLi), or are where the original geographical original collection locations are very close to one another (e.g., *X. helleri* Hx, Sara-or, Lance).

While there is no doubt that cryopreservation is an important resource for *Xiphophorus* germplasm, it is not the sole answer that will ensure the long-term existence of these species as necessary members of our planet's biological diversity. For that we will need to sustain our wild populations and support our living collections.

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New Chapter

Sperm Cryopreservation and Storage at the Zebrafish International Resource Center

Zoltan M. Varga and Monte Westerfield

Introduction

The late George Streisinger was the first to recognize that zebrafish *Danio rerio*, a tropical freshwater cyprinid, was ideally suited for genetic and embryological studies because it offers considerable experimental advantages (Streisinger et al. 1981). For example, embryonic and larval development are rapid (Figure 1) (Kimmel et al. 1995), a generation is relatively short in duration (3-4 months), and females produce hundreds of progeny. Additionally, zebrafish eggs are easily accessible for genetic and embryological manipulations and embryos lack pigment during the first 36 hr of development, which greatly facilitates the observation of organogenesis with single-cell resolution. Zebrafish have become popular in recent decades, and research extends into the fields of evolution, neuroscience, behavioral biology, immunology, cancer research, ecology, toxicology, drug screening and development, and several other disciplines.

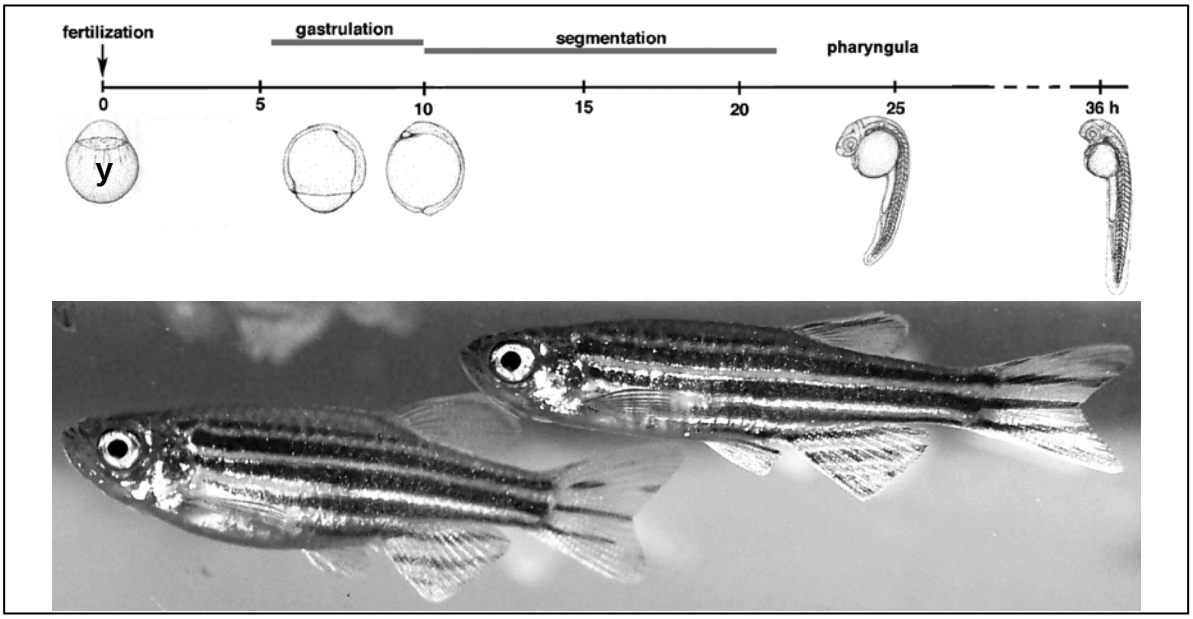

Figure 1. (Top) Timeline of zebrafish embryogenesis. Rapid development occurs in the first 36 hr after fertilization. During the pharyngula period (24 - 48 hr), the transparent embryo shares many characteristics with embryonic chick, mouse, and human pharyngulae. The yolk cell (y) is approximately 0.5 mm in diameter (sketches from Kimmel et al. 1995). (Bottom) Adult female (left) and male (right), these fish are ~3 cm from mouth to caudal peduncle.

Recent advances in molecular biology and cloning have led to the identification of thousands of zebrafish genes (Thisse et al. 2001). The roles genes play in regulating development and controlling metabolism have been characterized in genetic screens that have generated

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thousands of novel zebrafish strains (Driever et al. 1996, Haffter et al. 1996). In addition, several new technologies have become available, such as the generation of transgenic reporter lines, viral- and transposon-based insertions (Amsterdam et al. 1999, Driever et al. 1996, Haffter et al. 1996), TILLING (targeting induced local lesions in genomes) (Draper et al. 2004), enhancer or gene trap strategies (Davison et al. 2007, Ellingsen et al. 2005, Kawakami 2005), and recently, targeted knock-out of genes (Doyon et al. 2008, Meng et al. 2008). Sequencing of the zebrafish genome is being concluded, and recent estimates suggest that the genome contains approximately 23,000 protein encoding genes (*www.sanger.ac.uk/Projects/D_rerio*). Several laboratories, mutagenesis programs, and consortia continue to use this information and exploit new technologies to generate more unique fish lines (*www.zf-models.org*). Millions of research dollars (Table 1, next page) have been and continue to be spent on creating these genetic resources for biomedical research, and we can expect a significant increase in their number and diversity in the near future.

The Zebrafish International Resource Center (ZIRC)

The ZIRC was established in 1998 to provide a central repository for wild-type, transgenic, and mutant strains of zebrafish. Because a large, centralized facility can be cost-effective due to certain economies of scale, individual laboratories can more efficiently focus their resources towards research questions. Live adult fish are submitted to ZIRC by individual researchers and popular lines are maintained live so they are readily available. However, because the ZIRC facility has acquired more lines than it can keep alive, less frequently requested lines are cryopreserved. Upon request, these lines are revived by thawing and *in vitro* fertilization. The turnover between live and cryopreserved lines ensures that ultimately all lines acquired by the Resource Center are cryopreserved and resources that were generated using millions of research funds are available for future research.

The ZIRC also provides other materials for zebrafish research, such as monoclonal antibodies and cDNA expressed sequence tag (EST) gene probes. The ZIRC also offers a Pathology Service for diseased and healthy fish. A staff veterinarian analyzes fish and tissue samples and, in collaboration with a staff aquaculture specialist, provides advice about zebrafish health, husbandry, and disease prevention or containment. The ZIRC also maintains *Diseases of Zebrafish in Research Facilities, for Prevention, Diagnosis, and Treatment of Diseases Affecting Zebrafish*, an online manual that describes the most common diseases of laboratory zebrafish (*zebrafish.org/zirc/health/diseaseManual.php*). Additionally, the ZIRC staff develops general husbandry standards and procedures for maintaining healthy strains of zebrafish.

The key goal of ZIRC, to serve as a central repository and to preserve a maximum of resources for the research community, depends on its capacity to acquire, store, and redistribute as many lines as possible. Because physical space for live and frozen lines is limited, ZIRC needs to develop cryopreservation methods for its own purposes and for the research community at large to ensure that resources can be stored for future generations. To this end, ZIRC currently uses, with minor modifications, the cryopreservation protocol developed by Draper and Moens (Draper et al. 2004), which was adapted from the cryopreservation method used by Harvey et al. (1982). This method has the highest current throughput capabilities and therefore best suits the needs of ZIRC to cryopreserve large numbers of samples.

Table 1. Examples of past and current funding for zebrafish mutant and transgenesis screens. The table does not list additional funds provided to support long-term, ongoing genetic mutant screens or to generate enhancer trap lines, gene trap lines, or funds provided for generation of transgenic reporter lines.

Institution	Start year	Screen name and type	Approximate budget	Initially recovered lines or mutations
Max-Planck Institute, Tübingen, Germany (Haffter et al. 1996)	1996	ENU mutagenesis	US$ 3 million	1163
Massachusetts General Hospital; Harvard Medical School (Driever et al. 1996, Wolfgang Driver personal communication)	1996	ENU mutagenesis	US$ 2.5 million	695
Max-Planck Institute, Tübingen, Germany (Haffter et al. 1996)	2000	ENU mutagenesis	US$ 5 million	~ 2000
University Freiburg, Germany (Wolfgang Driver personal communication)	2001	ENU Mutagenesis Dopaminergic and pancreas mutants	US$ 0.3 million	46
Massachusetts Institute of Technology (Nancy Hopkins personal communication)	1996	Viral Insertion mutagenesis screen	US$ 8 million	~ 700
ZF-Models, European research consortium (Haffter et al. 1996)	2004 - Ongoing	Targeting Induced Local Lesions in Genomes	€ 12 million	~ 6000
Sars IMMB Bergen, Norway (Amsterdam et al. 1999, Ellingsen et al. 2005, Tom Becker personal communication)	2005	Mouse Retroviral Insertion Enhancer Trap	US$ 1.9 million	~ 400
Dept. Physiology University of California San Francisco (Davison et al. 2007, Herwig Baier personal communication)	2007	Transposon based enhancer trap	US$ 0.25 million	200

The ZIRC Facility

The Zebrafish International Resource Center is housed on the University of Oregon campus in Eugene, Oregon, USA. The recirculating water system consists of four water systems with fluidized sand biological filters (Aquaneering, Inc., San Diego, California, USA) and a total water volume of ~90,000 L. The facility holds as many as 400 live lines (Figure 2), which corresponds to about 80,000 fish/yr. Tank water is prepared from reverse osmosis water. Salts and buffers are automatically added to stabilize the pH and to maintain water conductivity. These and other parameters such as water temperature are monitored continually. To maintain biosafety, the facility has a quarantine room with a flow-through water system where all imported fish remain and are bred. Only surface-sanitized (bleached) embryos are transferred from the quarantine room into the nursery of the main fish room. Until recently, the facility provided space for only a relatively modest sperm bank with 3 liquid nitrogen freezers that hold as many as 27,000 samples of cryopreserved sperm. Currently, at least 10 samples need to be frozen per line; hence, the ZIRC sperm bank could hold at most approximately 2,700 lines. However at the rate new lines are being generated today, the Resource Center will soon run out of storage space. Therefore, the facility was recently remodeled to provide additional freezer space for up to 10,000 lines.

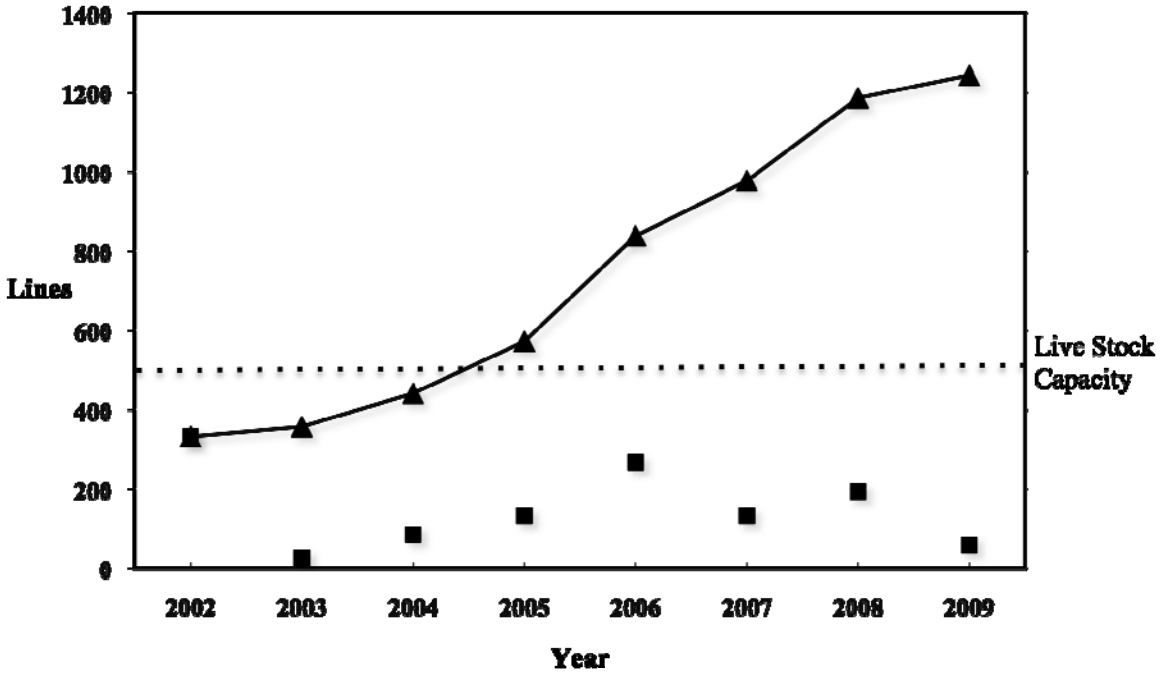

Figure 2. Cryopreservation improves the efficiency of zebrafish imports. Since 2001, more than 1,200 total lines (triangles) have been imported to ZIRC. Peaks in imported new lines (squares) in 2002 and 2006 occurred because of large-scale imports by shipping of cryopreserved sperm, indicating that this method is more efficient than importing live adult fish. The dotted line indicates the approximate capacity of ZIRC to maintain live lines. Importation of cryopreserved lines and cryopreservation as a line management tool minimizes the resources needed for fish maintenance.

Sample Collection at ZIRC

To establish a comprehensive, centralized repository, at least one allele of each locus should be acquired by ZIRC; however, usually several alleles exist at each locus. Additional reporter lines may also exist that express a fluorescent protein or an enzyme under the control of a particular promoter or enhancer. Multiple lines of a locus or variations of a transgenic line are accepted only if they provide interesting or useful features for research (for example, null alleles versus hypomorphic alleles). To maximize and streamline the number and variety of lines housed at the Resource Center, several guidelines have been established for acquisition, and an external advisory board helps prioritize lines for importation, making the acquisition process as time- and cost-efficient as possible.

To exclude redundancies, a record or a curator-approved name must be established with the Zebrafish Model Organism Database ZFIN (*www.zfin.org*). Second, the ZIRC collects information relevant to maintaining the fish lines (husbandry information), genotyping carriers of mutations or transgenes (if mapped or cloned), or identifying phenotypes. This information also helps to determine interest or importance of the submitted line for current or future research. Based on the expected or established popularity of a line (derived from publications or frequency of requests), the ZIRC staff decides whether an imported line should be maintained alive or be cryopreserved in the sperm bank. The prospective popularity of a line also determines how many frozen samples are stored in addition to the minimal number of samples to maintain while filling orders. A key prerequisite for import approval is whether the submitter can supply healthy breedable adults. Healthy, adults of breeding age maximize the ability of the staff to breed popular lines, establish live stocks in the fish facility, and freeze sperm in the quarantine facility. Recently, cryopreservation also became extremely useful for importing large numbers of lines in a time and cost efficient manner (Figure 2).

The Current Status of Zebrafish Cryopreservation

In spite of the significant costs associated with generating thousands of research lines, little effort has gone towards preserving them safely for future research. Typically, the capacity of laboratories to maintain strains is limited by personnel and space. Thus, without a concerted effort to bank them many lines are in danger of being lost. Currently, cryopreservation is the preferred method in a wide variety of taxa for preserving samples from endangered species, for maintaining genetic diversity of populations, and for storing rare or important genetic materials. Cryopreservation is the best alternative to live stocks of zebrafish for several reasons: 1) facility space is efficiently used, 2) the effective reproductive time of males is extended, 3) valuable resources are not lost if a line cannot be maintained live, 4) reverse genetic mutagenesis strategies, such as TILLING, are more feasible because live fish do not need to be maintained (Draper et al. 2004), and 5) cryopreservation is highly cost-effective because it saves much-needed funds for electricity, water, filtration, tank cleaning, repair, food, and personnel.

Unfortunately, the cryopreservation protocols currently in use by the zebrafish research community can be relatively difficult to learn and adopt in individual laboratories. Moreover, success rates and fertilization rates vary considerably among laboratories, most likely because intended or unintended modifications have occurred (Draper et al. 2004, Morris et al. 2003) to the procedure developed by Walker and Streisinger (Westerfield, 2007) based on the original method (Harvey et al. 1982). Moreover, some protocols still include the use of additives such as

powdered milk and storage containers such as glass capillary tubes and cryovials, which are inefficient and hamper necessary quality controls such as motility estimation and cell density measurements.

One study tested ethylene glycol, dimethyl sulfoxide (DMSO), glycerol, and dimethyl acetamide (DMA) as potential cryoprotective agents to replace methanol, which has cytotoxic effects and is known to mutate DNA (Morris et al. 2003). The second modification aimed at procedural optimization and led to a more streamlined protocol that can be more easily adapted to various laboratory conditions (Draper et al. 2004). However, both methods produce only modest improvements on the original protocol (Harvey et al. 1982) (Table 2) in terms of post-thaw fertilization rates, materials, and procedure. Without a solid understanding of the biophysical principles governing the physiology of frozen cells, these modifications have largely been tested empirically by determining whether or not they reduce or improve overall post-thaw fertilization rates. Indeed, it remains unclear whether the community currently uses the most practical, reliable, and efficient method. Recently, another variation omitting the use of powdered milk has been developed in a study where four cryoprotectants, DMSO, DMA, methanol, and glycerol, were tested (Yang et al. 2007). This study used 0.25-ml French straws which are compatible with automated equipment for filling and sealing and enable high-throughput processing. These methods work sufficiently well for laboratories specializing in cryopreservation, however there is insufficient information to allow them to be used in a robust manner across the breadth of the research community or, at present, for automated high-throughput applications.

Table 2. Current cryopreservation methods for zebrafish providing a cross-comparison of key steps and agents used in cryopreservation protocols.

	Published studies			
	(Harvey et al. 1982)	**(Morris et al. 2003)**	**(Draper et al. 2004)**	**(Yang et al. 2007)**
Cryoprotectant	10% methanol	10% DMA	8% methanol	8% methanol
Diluent	Ginsburg	BSMIS	Ginsburg	HBSS
Collection method	Squeezing	Dissection	Squeezing	Dissection
Container	Capillary	Capillary	Cryovial	0.25-ml Straw
Extender	HBSS	BSMIS	HBSS	HBSS
Freezing method	Dry ice	Dry ice	Dry ice	C-R freezer
Freeze time	30 min	30 min	20 min	8.5 min
Freeze rate	16 °C/min	?	?	10 °C/min
Motility	43 ± 12%	12 ± 6%	?	78 ± 10%
Thawing	Air	37 °C	33 °C, 8 sec	40 °C, 5 sec
Fertility	*51 ± 36%	14 ± 10%	28 ± 18%	33 ± 20%

Reported as percent hatch which may be different from percent fertility in other columns.

Abbreviations: N,N, dimethyl acetamide (DMA), Hanks' balanced salt solution (HBSS), buffered sperm motility-inhibiting solution (BSMIS), Controlled-rate (C-R).

Variability in Post-thaw Fertilization

Several factors may contribute to the variable outcome of post-thaw fertilization, including (but not limited to): skill level and proficiency of individual researchers, materials used in different laboratories or between different freezing events, male variability, variations in sperm sample volume, variations in cell density, variations in sperm quality, variations in freezing rates, egg quality during *in vitro* fertilization, and even the definition of successful fertilization, which can range from sperm-egg penetration, to first cell division, to development until 24 hr, hatching, or inflation of the swim bladder. To accurately predict sample reliability and, therefore, the probability of recovering valuable research lines, current techniques including thawing and *in vitro* fertilization need to be improved further and standardized.

Considerations for Future Zebrafish Cryopreservation

After three decades of highly successful research, zebrafish science is faced with unique challenges that result from this success. For example, the sharp increase in the number and diversity of zebrafish strains is so large that laboratories have difficulty keeping up with banking. In the absence of concerted efforts, many of these lines are in danger of being lost; therefore, preservation has to occur as a collaborative effort on several levels - in individual laboratories for the interim and at the Stock Center for the long-term. The ability to cryopreserve sperm reliably with predictable post-thaw fertilization rates is critical, because even the most successful methods are still highly variable and thus affect the overall success rate of recovering frozen stocks. Several strategies must be pursued to optimize and standardize zebrafish cryopreservation.

Standardization of Protocols and Terms

In 2005, the ZIRC, in collaboration with the Smithsonian Institution and the Louisiana State University Agricultural Center, hosted a symposium on zebrafish line cryopreservation at which several examples of discrepancies became apparent (*Resolving Sperm Cryopreservation Issues for the Zebrafish Research Community*; West Coast Zebrafish Meeting in Eugene, Oregon). One problem was that common cryobiological terms are used differently in reference to laboratory standards. For example, the absence of quality controls and standardized concentrations of frozen sperm samples leads to poor characterization of fertility which is compounded by different definitions of fertilization rates when eggs are fertilized *in vitro* from frozen sperm. As a standard, we propose specification of: 1) extender, components, and their concentrations (molarity or mg/L); 2) final cryoprotectant concentration (percent, v/v) after mixing with sperm; 3) final sperm concentration (cells/ml) in cryoprotectant; 4) freezing container type and volume; 5) freezing rate (in ºC/min); 6) storage container, freezer type, and storage time in liquid nitrogen; 7) sample thaw temperature, or thaw rate in ºC/min; 8) *in vitro* fertilization solutions, concentrations, and volumes (sperm and egg concentration in water at time of fertilization); 9) total number of healthy, normal eggs at time of fertilization; and 10) total number of live embryos during the zygote period and cleavage stage (0.75 to 2.25 hr).

Similarly, many terms currently used in zebrafish cryopreservation need to be defined consistent with specific and standardized terms used in cryobiology. For example, the term “success rate” should not be confused with fertilization rate. Rather, it should be reserved for the overall success when regenerating a particular line from frozen samples.

Establishment of Consistent Quality Controls

The relatively small sample size (1-2 µL) and the current practice of adding powdered skim milk prevent assessment of important quality control parameters such as cell motility, morphology, density, and total cell count. Current protocols also specify that sperm should be on dry ice within 30 seconds after adding cryoprotectant. This time constraint makes it difficult, if not impossible, to assess sample quality before freezing. Further research into the best practices for quality control is necessary. A recent study showed that exposure time to the cryoprotectant may be extended for 10 min or longer without impact on sperm motility and cell survival (Yang et al. 2007), providing additional time for quality control.

Without assessment of important quality control parameters such as cell density, it is difficult to predict post-thaw fertilization rates. It was found that a final dilution of 10^8 cells/ml yielded the highest post-thaw fertilization rates, making the use of powdered milk unnecessary (Yang et al. 2007). In the Draper-Moens method, cryoprotectant concentration is normalized at the expense of varying cell densities. To reduce further post-fertilization variability, cell density and cryoprotectant each need to be adjusted to optimal concentrations.

Improvement of Efficiency and Consistency by Automation

Currently, zebrafish are processed individually to obtain and freeze sperm samples. Thus, each male needs to be netted, anesthetized, and blotted to collect samples which are placed in cryovials and frozen. Each step requires handling of individual fish, the use of various materials, scissors, containers, and changes of instruments, all of which consume significant amounts of time. To speed the process, some samples are frozen while milt is collected from other males. This approach necessitates considerable effort for accurate bookkeeping of sample collection times, coordination, and logistics. A pivotal goal will be to streamline the entire protocol so that key steps in the protocol become more efficient. For example, sperm can be stored in a salt solution extender on ice for several hours. Thus, collecting sperm from all males and storing it in extender on ice could be the first step, adding cryoprotectant to all sperm samples would be the second, and batch freezing would be the third. Automated straw filling and batch freezing in controlled-rate freezers is already used for farm animals such as dairy cattle (Lang et al. 2003). However, such technologies have yet to be developed for small species such as zebrafish, although they would be highly desirable for high-throughput applications in the Resource Center, for example. Automation of key steps in the protocol will also ensure that critical freezing parameters are reproduced more consistently.

Expansion of the Types of Cryopreserved Materials

Cryopreservation of germplasm is not limited to sperm. In April 2007, the National Center for Research Resources of the National Institutes of Health held a meeting entitled *Achieving High Throughput Repositories for Biomedical Germplasm Preservation Workshop* (*www.esi-bethesda.com/ncrrworkshops/Biomedical/index.aspx*). Among other issues, it was noted that for other research organisms, various types of cells and tissues have been successfully cryopreserved. In addition to preserving research resources, several techniques such as cell or tissue culture, cell or tissue transplantation, and cloning are supported by the ability to cryopreserve and regenerate various cell types. In mouse, rat, swine, and non-human primates, spermatozoa, oocytes, ovarian tissue, testicular tissue, embryonic stem cells, and even embryos have been successfully cryopreserved. In contrast, zebrafish and most other aquatic species are currently limited to the cryopreservation of sperm (i.e., only the male genome), which is a

considerable genetic and experimental disadvantage. Only recently have modest advances been made in cryopreservation of zebrafish blastomeres followed by restoration through germline chimeras (Lin et al. 2009). Thus, developing efficient methods for cryopreserving a variety of cells and tissues including embryonic stem cells, somatic cells, and primordial germ cells is a necessity for future zebrafish research.

Collaboration on Large-scale Imports

Typically, the acquisition of large numbers of live lines by the Stock Center is associated with high costs for frequent shipping and materials over extended periods. Because space is limited at the submitting fish facility and at the Resource Center, significant logistical and financial constraints also arise on both ends due to increased demands on quarantine room space, personnel coordination, and operational costs. We have found that cryopreservation makes large-scale imports more time and cost efficient. To make this possible, ZIRC staff can travel to the submitting laboratory, cryopreserve fish lines within a few d or weeks, and ship cryopreserved sperm samples to ZIRC for storage and distribution. Thus, the Resource Center can import lines more rapidly by focusing efforts on cryopreservation during this relatively brief period. Although costs for special shipments, travel, and housing for staff members are incurred, as well as assistance from a technician at the hosting laboratory, these costs are small compared to live imports that take up personnel time and maintenance of live resources in both places over extended periods of time. Alternatively, ZIRC also provides cryopreservation training for the personnel of the submitting laboratory and shipping of frozen samples to the Resource Center can then occur according to project schedules. Therefore, importation of large numbers of cryopreserved lines as a coordinated, collaborative effort between the submitting laboratory and the Resource Center pays off rapidly, because a large number of lines can be imported and made available in a relatively short amount of time.

Presently, laboratories do not calculate costs for Resource Center submissions into their research grants. However, the NIH expects that the submitting laboratories will carry Center acquisition expenses. Hence, researchers who intend to generate large numbers of lines and make them available to the research community need to factor resource submission costs into their grant budgets. To this end, the ZIRC can be contacted informally to advise on prospective submission costs and to provide letters of support to funding agencies.

Expansion of Resource Center Storage Capacity

When construction of the Zebrafish International Resource Center was completed in 1999, several genetic screens had been conducted and several hundred lines existed. However, molecular techniques were not as advanced at that time, and since then, thousands of additional novel lines have been generated, including wild-type, mutant, and a variety of transgenic lines. Thus, the number of lines generated quickly exceeded expectations, and the space constraints to maintain them alive or cryopreserved at the ZIRC soon became evident (Figure 1). Currently, ZFIN lists 9,722 fish lines, and 1,253 of these have been acquired by the ZIRC (as of November, 2010) either as live adults, or as cryopreserved stock. Indeed, cryopreservation has become the central management tool to deal efficiently and effectively with the increasing numbers of lines. Less popular live lines are regularly “retired” to the sperm bank and samples are revived upon request. This turnover has steadily helped to increase the number and variety of lines housed at the Stock Center without creating a burden on the capacity for live lines. However, due to space constraints, even freezer space has become limited at the Resource Center.

As stated above currently, approximately 2,700 lines can be stored; however, at the rate new lines are being generated, this space is expected to run out in the near future. To ensure the mission of ZIRC as the central repository for zebrafish lines, it is critical to develop additional storage options. Part of the solution was to remodel and expand cryopreservation space. However, the Resource Center also needs to disseminate information about cryopreservation, train laboratory staff at workshops and conferences, and provide cryopreservation information and methods to the public in general. This will enable laboratories to establish their own cryopreservation programs. Laboratories will then be able to generate large numbers of lines without fear of losing them. By cryopreserving their own stocks, researchers will be able to prioritize their research projects, retain fish lines for much longer, and submit lines selectively to the Resource Center when projects are published. Distributing the storage problem across multiple facilities will also take pressure off the Resource Center, because submitted lines will have been selected for scientific success and the volume of submissions may be somewhat smaller, albeit extended over longer periods.

A New Role for Zebrafish in Cryopreservation

Zebrafish have been successfully used as a laboratory model organism in a wide variety of biomedical disciplines. However, their relatively small size also poses problems for some applications. For example, cryopreservation is difficult because sample volumes average around only 1-2 µL per male. However, this apparent constraint can also be an advantage. As for so many other scientific disciplines, zebrafish could serve as a model organism to explore new cryopreservation strategies, technologies, and methods for small species in general, such as insects including *Drosophila*. Pioneering cryopreservation in zebrafish would provide benefits to preserve small organisms from extinction or to establish similar cryogenic repositories for other research organisms.

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New Chapter

Medaka Cryopreservation and the Aquatic Biotechnology and Environmental Laboratory

Richard N. Winn

Introduction

Over the past two decades, the application of small fish species as animal models in biomedical and environmental research has increased dramatically. Among these fish, species such as the medaka *Oryzias latipes*, mummichog *Fundulus heteroclitus*, swordtails and platyfish *Xiphophorus* spp., and zebrafish *Danio rerio*, have been embraced for various attributes such as short generation times, cost-effective culture, compact genomes, and ease of manipulation to be exploited in a wide range of disciplines ranging from genetics and developmental biology to toxicology and cancer research. Coincident with the emergence of fish among the leading animal models, has been an increasing demand for resources, including access to stocks of wild-type, inbred, transgenic, and mutant fish lineages. In addition, stimulated by expanding research using fish as the "white mice with fins", there have been significant efforts to improve all aspects of fish care and use to achieve standards approaching those applied to the widely used mammalian models, such as development of well-defined diets, disease monitoring, genetic testing, and construction of specialized aquatic facilities to support optimal culture and husbandry.

This chapter summarizes recent advances in research using medaka with emphasis on how these advances have stimulated efforts to improve procedures for germplasm cryopreservation for this growing research community. An overview is also presented on the Aquatic Biotechnology and Environmental Laboratory (ABEL), a specialized aquatic animal facility that serves as a resource center to support the culture, development, and application of fish in research.

Recent Advances in Medaka Research and Cryopreservation Needs

Medaka is a small egg-laying freshwater fish native to Asia, primarily in Japan, but ranges to Korea and eastern China (Wittbrodt et al. 2002). The preferred habitat is rice *Oryza sativa* fields as reflected in the common and scientific names as rice fish (Jordan and Snyder 1906). Numerous aspects of medaka biology, including physiology, genetics, and embryology have been studied for more than a century (Yamamoto 1975). Medaka has various attributes including, ease of culture, short generation time (4-6 weeks), prolific capacity for reproduction (>2,000 eggs/female/breeding cycle), transparent embryos, amenability to transgenesis, wide tolerance of temperatures (4 to 40^{o}C) and culture conditions, low rates of spontaneous tumor formation, and responsiveness to a wide range of toxicant exposures, which afford significant benefits in the dual roles medaka plays as an animal model for biomedical and environmental research (Hawkins et al. 1988, Ishikawa 2000, Wittbrodt et al. 2002, Yamamoto 1975).

Medaka is one of the best known fish in Japan, and has been bred by hobbyists for at least 200 years (Ishikawa 2000). The culture of fish in large commercial pond operations has benefited researchers by providing novel spontaneous hybrids, mutants, and wild- type strains.

Winn, R. 2011. Medaka Cryopreservation and the Aquatic Biotechnology and Environmental Laboratory. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 820-827.

In particular, access to these large populations over decades have assisted researchers in collecting, characterizing and maintaining over 80 visible-phenotype mutants at the medaka bioresources center at Nagoya University (Ozato and Wakamatsu 1994, Tomita 1992).

Overall, recent advances in genomic and genetics research have intensified interest in medaka as a comparative animal model. In particular, with the completion of the first draft of the medaka genome sequence and subsequent releases of additional genome coverage (Kasahara et al. 2007), medaka is placed among the leading model organisms for genomics and developmental genetics research. Recently, successful procedures for disruption or knock-out of the function of specific genes have been demonstrated in medaka (Taniguchi et al. 2006). In this approach referred to as TILLING (Targeting Induced Local Lesions in Genomes), random mutations are introduced in the male genome using the germ cell mutagen N-ethyl-N-nitrosourea (ENU) followed by breeding with wild-type females to establish a library of genomic DNA and sperm from the F_1 generation male offspring. Using DNA sequence information for the gene of interest, genomic DNA from the library is sequenced to identify individuals carrying specific mutations. These targeted gene mutants are retrieved using *in vitro* fertilization with the cryopreserved sperm, and lineages are established by subsequent outcrossing. The generation of gene knockout models by target-selected mutagenesis represents a significant achievement as it provides the means to develop new lineages of mutant medaka for virtually any gene of interest to explore the mechanisms of gene function and related disease processes.

The continued expansion of the genetic resources for fishes including specialized lineages such as targeted gene knockouts and transgenics will contribute thousands rather than hundreds of new lineages of fish over the next several decades. In addition, increased recognition for the needs to conserve diminishing native populations of fish, further emphasizes the need to improve standardization, efficiencies and high-throughput cryopreservation of stocks.

The Aquatic Biotechnology and Environmental Laboratory

The Aquatic Biotechnology and Environmental Laboratory at the University of Georgia is an international aquatic resource center focused on research using medaka and mummichog (Hawkins et al. 2001). The ABEL is a multi-purpose facility that combines aquatic animal culture laboratories with modern molecular and toxicological laboratories to meet the needs for: 1) fish culture, 2) development and application of transgenic and mutant lineages, and 3) research on mutagenesis and environmental toxicology.

Fish Culture

Small fish species offer significant practical benefits for maintaining laboratory populations at lower costs compared to mammalian models. However, as culture of aquatic animals has changed, emphasis from primarily short-term holding of animals collected from the wild to long-term perpetuation of specialized laboratory populations, it has become more apparent that improvements in aquatic animal resource facilities as well as in standard procedures are needed to optimize efficiencies in husbandry. Unlike some aquatic facilities of the past that were converted from storage or bench laboratories, ABEL was designed specifically for aquatic animals guided by the goals to optimize the long-term culture of wild-type and specialized strains, and to integrate standards of animal care and use equivalent to those applied to mammalian models.

Among the design features, ABEL contains four separate culture laboratories, each with dedicated climate control and lighting to provide standardized procedures to support maintenance of stocks and to provide flexibility to meet changing research needs. The facility is equipped to maintain fish using de-chlorinated municipal fresh water or artificial seawater in either specialized self-contained recirculating systems or standard aerated aquaria. In addition, to meet a primary research mission in using fish as models in environmental toxicology, multi-purpose wet-laboratory space and specialized exposure equipment are used to study responses of fish to various chemical exposures under highly controlled conditions (Figure 1).

Figure 1. Examples of the recirculating culture systems located at ABEL.

Specialized Strains, Mutants and Transgenic Lines

The ABEL serves as a central animal resource and distribution center for wild-type, mutant and transgenic strains of medaka developed at the facility, and as a repository for specialized strains developed by other researchers. Currently, there are at least 30 research laboratories active in medaka culture within the United States. Live animals are provided to researchers at minimal cost upon request and collaborations are encouraged. The ABEL currently maintains a small fraction of the strains identified and developed world-wide, although significant additions to current stocks are anticipated over the next several years. The most commonly used wild-type strain in the USA, the orange-red or CAB strain (Carolina Biological) originally derived from a southern Japanese strain, and the HNI strain, derived from a Northern Japan wild-type strain, are among the strains in continuous culture at ABEL. The transgenic lineages include the λ transgenic and pUR288 transgenic medaka used for *in vivo* mutagenesis research (Winn et al. 2001, Winn et al. 2000). Mutant strains include the radiation-sensitive strain (RIC 1), and the specific-locus test strain for analysis of germline mutations (T-5) (Shima and Shimada 1991) and the targeted gene knock-out strains for the Rev1 gene (provided by T. Todo, Osaka University) and the P53 gene (Taniguchi et al. 2006).

Environmental Mutagenesis and Toxicology: λ Transgenic Medaka

Notable among the strains maintained at ABEL, the λ transgenic medaka is one of the most extensively studied and widely applied transgenic fish models in biomedical and environmental research (Winn 2006). The λ transgenic medaka, based on using mutation target genes harbored within the bacteriophage λLIZ vector, was developed to address the need for improved approaches to detect mutations in whole animals (Winn et al. 2000). The most widely used transgenic mutation assay in mice and rat models is also based on the λ vector, thereby providing unprecedented opportunities for comparative studies of mutagenesis in distantly related organisms (Figure 2). Similar mutation assay procedures are used for the fish and rodents whereby mutations in the target genes are detected in the tissue of interest by recovering the bacteriophage λLIZ vector from the genomic DNA and using specialized strains of indicator bacteria to distinguish mutant from non-mutant genes.

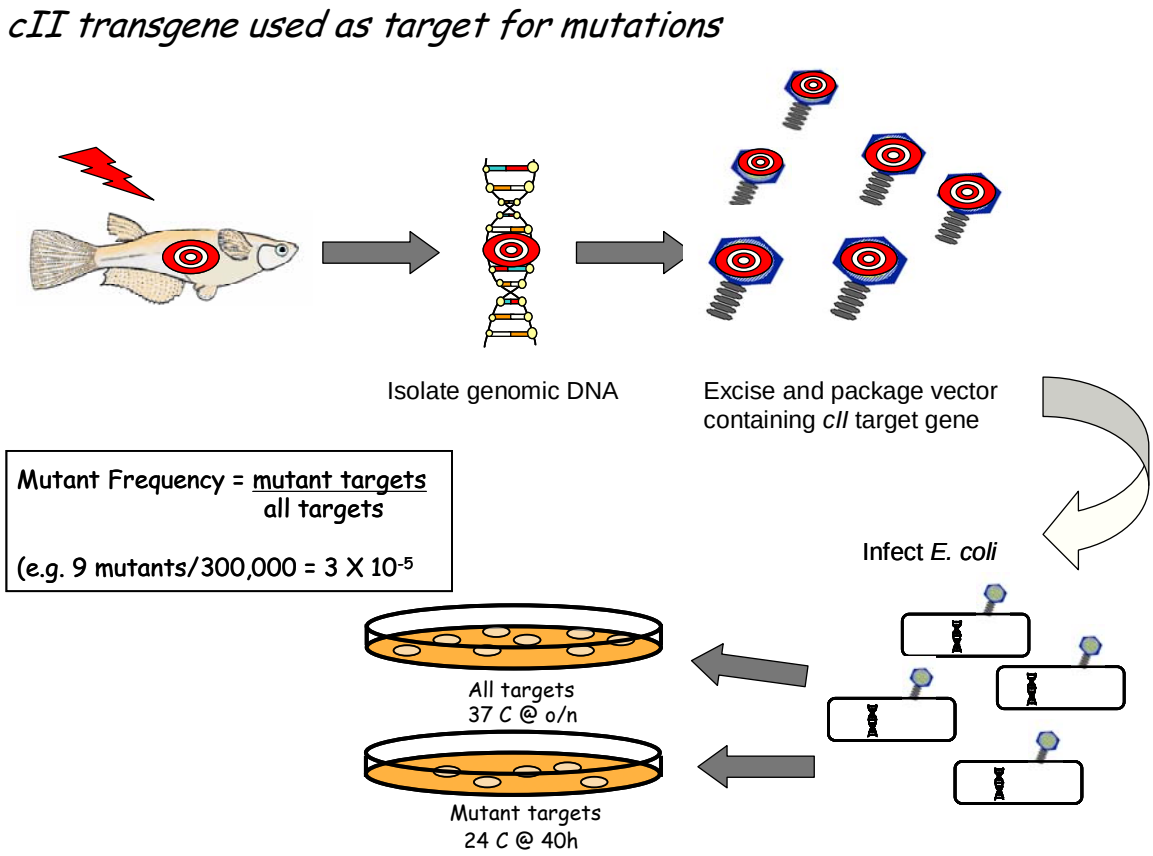

Figure 2. Illustration of the *cII* mutation assay using the λ transgenic medaka. After mutagen treatment and sufficient time for mutations to manifest, genomic DNA is isolated. The λ bacteriophage vector is removed from the fish genomic DNA and packaged as individual phage particles which infect the G1250 *E. coli* host. λ phage with a mutation in the *cII* are selected by forming plaques while phage containing wild-type *cII* are indistinguishable on the *E. coli* lawn. The *cII* mutant frequency is the ratio of the number of mutant λ *cII*- plaques selected to the total number of plaques screened.

Using a variety of chemical and physical mutagens and exposure regimens, adult, juvenile or embryos of λ transgenic medaka have been applied in studies ranging from chemoprevention (Winn et al. 2005), comparative chemical carcinogenesis and mutagenesis (Hobbie et al. 2009, Winn et al. 2001) and ultraviolet radiation (Winn et al. 2005), to health risk assessment of drinking water disinfection by-products (Geter et al. 2004, Winn et al. 2006) and assessment of a variety of contaminants in aquatic environment (Cachot et al. 2007, McElroy et al. 2006). Most recently, by demonstrating that chemically induced mutations in male parents are transmitted to progeny, and that the procedures for detected mutant offspring are practical, efficient, and sensitive, the λ transgenic medaka meets the requirement as a new animal model for study of germline mutations (Winn et al. 2008). Combined, these studies illustrate that fundamental mechanisms of mutagenesis are highly conserved among these divergent species and that fish provide new insights into processes of DNA damage and repair shared among fish and rodent models, and possibly humans.

Stock Centers as Germplasm Repositories

Projected Needs and Problems

As the applications for fish as animal models have expanded, so has the recognition of the practical challenges of husbandry and maintenance related to space, preservation of valuable stocks, and efficient exchange of animals and reagents. Projections of the numbers of new medaka lineages being developed vary, but the numbers of new strains and lineages may eventually reach into the thousands. Aquatic animal laboratories vary widely in sophistication and capabilities, ranging from simple rooms used by single investigators, to highly specialized facilities used by teams of researchers, or as a resource center for the broad research community. Most researchers discover the truism that the need for more animal culture space will eventually expand beyond what is available. Further, despite the significant cost advantages of maintaining small fish compared to that of mammals, husbandry of aquatic animals represents a significant investment, such that improving efficiencies of culture, reducing numbers of animals, and reducing risks of catastrophic losses of invaluable lineages due to accidents or disease remain important goals.

Research Needs

Medaka shares many attributes with the more widely used zebrafish, such as small body size, prolific capacity for reproduction, and transparent embryos that have contributed to these species being excellent animal models for developmental biology and genetics. Medaka has additional features of biology, such as small genome size, wide tolerance to temperature extremes, inbred lineages, and Y-linked sex inheritance that are gaining additional attention. Many of the challenges that currently confront the zebrafish research community have parallels in the medaka research community. In particular, as a consequence of recent research advances and resource demands, the maintenance of growing numbers of lineages as live populations has become more challenging than ever. Although the medaka research community has benefited from cryopreservation procedures that have become available over the last decade, sperm cryopreservation is not commonly practiced for medaka in the USA (Aoki et al. 1997). It is apparent that improvements in cryopreservation will be of significant benefit to the global medaka community. Among these benefits, efficient and reliable cryopreservation procedures will reduce the risks of catastrophic losses by disease outbreaks, accidents, natural disasters, and

breeding failures. Cryopreserved stocks will assist in maintaining large gene pools of selected strains, reducing the amount of space needed to maintain live animals, and increasing efficiencies in regional and international distribution of material.

Two recent articles have been published addressing medaka cryopreservation. The first (Yang and Tiersch 2009) characterizes motility activation and describes a unique combination of factors in medaka which may also be characteristic of euryhaline fishes. The second (Yang et al. 2010) describes a simplified and standardized protocol that can be adapted for use with automated equipment to fill and seal straws for high-throughput processing. To ensure that sperm cryopreservation is a reliable, cost-effective tool for securing and preserving genetic stocks, the overall process needs to be improved, with an emphasis on being highly integrated and comprehensive. The platform for the cryopreservation should integrate genetic and biological databases, long-term storage capabilities, inventory management, quality control, sample distribution pathways, biosafety assurance, utilization, and disposal practices. Such improvements in cryopreservation will provide a broad and growing number of researchers full access to the diverse attributes of medaka.

Acknowledgments

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New Chapter

Commercial Application of Fish Sperm Cryopreservation for Management of Genetic Resources

John T. Buchanan and Valérie Barbosa-Solomieu

Introduction

One driver that may facilitate the commercial adoption of fish sperm cryopreservation is the development of "value added" genetics. As aquaculture increasingly employs selective breeding (Rothschild and Ruvinsky 2007), genomics (Rise et al. 2007), and transgenic (Hulata 2001) approaches to improving productivity and commercial value, distinct lines of fish with defined phenotypes will be developed that have economic value. Cryopreservation technology is a logical application when considering how best to extract value from these lines of fish.

AquaBounty Technologies (*www.aquabounty.com*) is an example of a company focusing on "value added" genetics for aquaculture. We have characterized a line of transgenic Atlantic salmon *Salmo salar* (Du et al. 1992) for commercial application. These salmon possess a single integrant DNA construct containing a growth hormone cDNA (from chinook salmon *Oncorhynchus tshawytscha)* under the control of regulatory elements from an antifreeze protein gene (from the ocean pout *Zoarces americanus)* (Yaskowiak et al. 2006). This line of fish grows significantly faster, reaching market size in about half the time of conventional salmon. Significant resources have been devoted to systematically addressing scientific questions relevant to evaluation by the U.S. Food and Drug Administration (FDA) of this line. Exhaustive studies assessing the safety, effectiveness, and composition have been completed and submitted for FDA evaluation. Further refinement of transgenic performance continues, utilizing genomic resources for selective breeding and producing defined lines within the transgenic population.

Under the conditions of the pending FDA-review, the transgenic salmon will be sold as all-female triploids to producers for grow-out in FDA-approved, physically contained, land-based cultivation systems (Figure 1, next page). AquaBounty is a genetics company, selling eyed-eggs to producers. In this context, our product is similar to other improved lines of fish under development; the underlying genetics that will be distributed to producers are the major component in the value of a company. These genetics are primarily stored as broodstock as new generations of brood are propagated. Cryopreservation of sperm has great potential for facilitating archival, distribution, and enhancement of in-house genetics.

Accordingly, we see four major areas of need in which sperm cryopreservation can play a large role in commercial aquaculture genetics:

1. Archiving of key genetics from defined lines. Cryopreservation would allow for storage of genetics from founder animals and representatives of each successively improved generation. The value of storing and having access to archived genetics is important in preserving a resource that can be used to address new problems such as disease sensitivity, and allowing reconstitution of lines from a catastrophic loss.

2. Providing backup milt from commercial males. To protect commercial operations from unforeseen losses, sufficient milt from broodstock to fertilize commercially relevant numbers of

Buchanan, J. T. and V. Barbosa-Solomieu. 2011. Commercial Applications of Fish Sperm Cryopreservation. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 828-833.

eggs could be cryopreserved. This would allow continuation of commercial function while working to restore broodstock populations.

3. Facilitating hatchery function and selective breeding programs. Cryopreservation could reduce the number of live males needed for spawning, freeing space and resources. Cryopreserved sperm could also be used to efficiently make specific crosses relevant to breeding programs, even repeating use of key males outside of the lifespan of the animal.

4. Enabling commercial application of cryopreserved milt. Cryopreserved sperm of sufficient quality could be used for fertilization of commercially relevant numbers of eggs. In-house, this would allow milt from desirable males to be cryopreserved independently of commercial production. This would make use of milt more efficient, and provide a temporal buffer between acquisition of commercially valuable milt and fertilization of eggs for commercial production. The ability to produce eggs for sale would be independent of the number of mature genetically improved males available, but would instead be dependent on the more tractable inventory of cryopreserved sperm. Finally, quality of cryopreserved sperm could be verified in advance of commercial application.

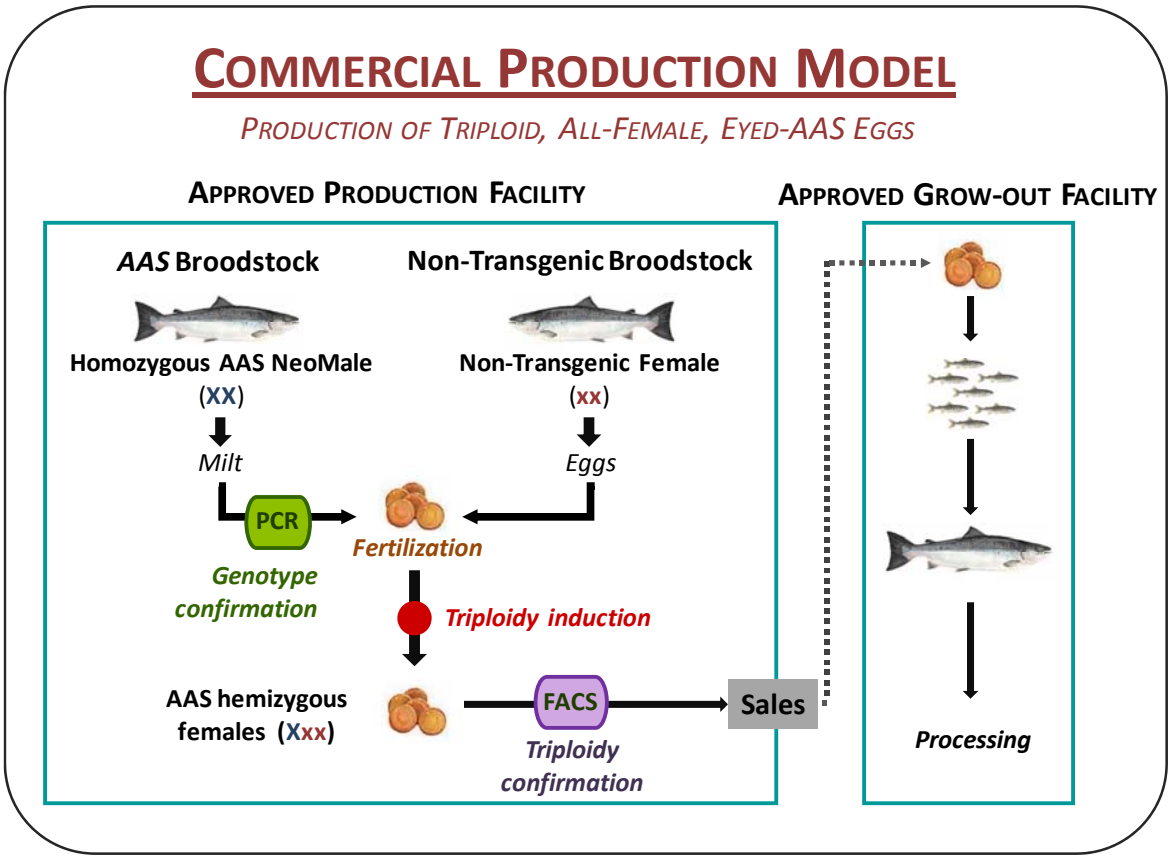

Figure 1. Commercial production model for AquAdvantage Salmon (AAS). Approved facilities will purchase all-female transgenic triploid eggs for grow-out and distribution. PCR: polymerase chain reaction; FACS: fluorescence activated cell sorting (flow cytometry).

We have considered how these four areas of need would be applied at AquaBounty (Table 1). For simple applications like archiving of key genetics, highly refined cryopreservation techniques are not a prerequisite. Freezing sperm from a relatively small number of males (e.g., 20) at quality sufficient to simply produce fish representative of the line of interest (e.g., 10% of fresh sperm fertilization rate) would be sufficient. For commercial applications like replacing use of fresh sperm in the hatchery, or using cryopreserved sperm to drive commercial production (Figure 1), it will be important to adapt cryopreservation techniques to larger-scale application. Cryopreserved sperm from each male would need to be effectively equivalent to fresh sperm to justify the investment in cryopreservation technologies. Poor quality sperm used on a commercial-scale would result in significant numbers of wasted eggs, as well as poor use of incubation space occupied by unfertilized eggs. Finally, for these commercial applications, not only must sperm be of high quality, but high-throughput methods for freezing sperm must be considered. For each male, we estimate freezing of 50 standard 0.5-mL straws. Freezing of sperm from 300 males would thus require 15,000 straws. For this number of straws, some automation of the freezing processes will be necessary.

Table 1. Areas of need at AquaBounty Technologies for commercial-scale sperm cryopreservation.

Needed capability	Number of donor males	Required fertilization capacity	Minimum required sperm quality	Comments
1. Archive genetics	50	1000 eggs/ male	Marginal: 10% of fresh sperm fertilization	Add 20 new males annually.
2. Backup commercial production	150	20,000 eggs/ male	Moderate: 50% of fresh sperm fertilization	Expand capacity as commercial demand grows. Add at least 50 males annually.
3. Use for hatchery and breeding	40	40,000 eggs/ male	High: 90% of fresh sperm fertilization	Add 20 males annually to replace used milt.
4. Commercial application	300	40,000 eggs/ male	High: 95% of fresh sperm fertilization	Freeze to replace used milt, improve existing milt, and expand capacity. Add at least 100 males annually.

Two models can be considered in establishing commercial-scale cryopreservation for fish. The first is construction of in-house freezing centers (Caffey and Tiersch 2000). The second is the development of centralized freezing centers with commercial-scale capabilities (Lang et al 2003) (Figure 3). We favor the second option as it ensures economies in capital costs and expertise that contribute towards cost-effectiveness. A large repository center can afford to purchase automated high-throughput equipment and to have highly skilled staff with a strong understanding of cryobiological fundamentals. Standardized quality control measures and database tracking of samples would be easier to implement at a centralized facility. It has, in addition, the possibility of adopting the systems and procedures that are needed to guarantee the

security of cryopreserved material. In this respect, centralized long-term storage facilities would ideally be large enough to allow for storage of cryopreserved materials in two separate locations. Finally, a single facility encourages better uniformity and rigor in record keeping (Mazur et al. 2008). A requirement for the centralized freezing center model, however, is that remote collection and shipping procedures yield sperm in suitable condition for cryopreservation.

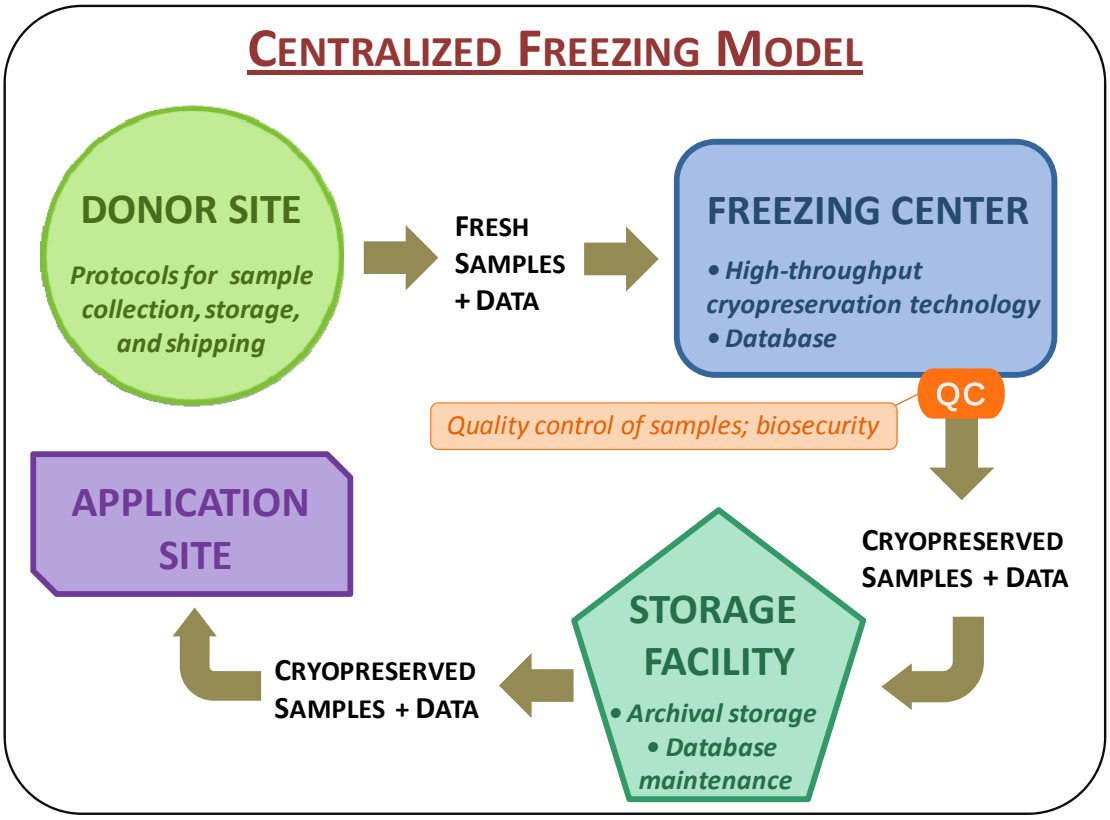

Figure 3. Centralized freezing model showing the flow of samples from the Donor Site to the Freezing Center, Storage Facility, and final Application Site. Quality control and biosecurity are the primary responsibility of the Freezing Center.

The centralized freezing model is based upon the existence of separate locations specialized in different steps of the cryopreservation cycle. The Donor Site (usually the client) must be proficient in sample collection, refrigerated storage (with or without the use of extenders) and shipping of fresh samples. The Freezing Center has the protocols, technology, personnel and supplies necessary for efficient freezing of sperm. Selection of straw volume, automation of straw filling, and the use of large cooling chambers would be required for commercial-scale cryopreservation of fish sperm. Systematic quality control must be in place to ensure that the frozen samples meet the standards required by the client. Sufficient space and equipment must be available for temporary storage of the frozen gametes. Frozen samples are subsequently shipped to a semi-permanent Storage Facility where adequate redundant storage of the samples minimizes the risks of loss of an entire batch of samples. Due to their considerable commercial value, redundant measures also need to be in place to guarantee the security of proprietary genetics in the Freezing Center and the Storage Location. Physical barriers along with controlled access should be implemented.

For this model to be functional, an efficient and streamlined process for information tracking and updating is necessary. A centralized database is required for accurate and timely integration of the information provided by the Donor Center (Male identification inventory and genotypic or phenotypic characteristics), the Freezing Center (number of straws, freezing parameters, quality control) and the Storage Facility (storage location). Database maintenance and data management would be best served as the responsibility of the Freezing Center. Data should be made available at any given point in time to any of the components of the system as part of quality control monitoring. In addition all sperm quality parameters recorded during the cryopreservation process would preferably be linked to: 1) any genomic information available for a given male, 2) performance of the male and progeny, and 3) fertilization data after straws from a male are used. This will allow rational identification of superior samples and removal of inferior samples from storage.

A final consideration would be biosecurity. Without adequate monitoring, transportation of frozen gametes has the potential to facilitate pathogen spread (Tiersch and Jenkins 2003). Strict biosecurity control is necessary to eliminate any risk of contamination of "clean" samples or cross-contamination of infected samples during the freezing process. A point to consider is whether to assume that all incoming sperm is potentially infected, and systematically apply procedures to reduce or eliminate infectious load; this would considerably increase sample processing costs (Mazur et al. 2008). Additional testing on thawed material may also be necessary when considering transfer of gametes across state and national boundaries.

This chapter focused on concepts for consideration in moving fish sperm cryopreservation from boutique, research-scale applications to high-throughput, commercial-scale approaches appropriate for industrial application. Given the great progress in fish sperm cryopreservation in the last 20 yr, we believe research focused on transfer of laboratory-based techniques for cryopreservation to commercial-scale applications is warranted. As a final note, the best case for application of cryopreservation would include freezing and storage of eggs, embryos, and sperm. Other than success with oyster oocytes (Tervit et al. 2005) and larvae (Paniagua-Chavez et al. 1998), almost all successful applications of cryopreservation have been applied to fish sperm only. Success in preservation of reconstitutable fish genomes would be a significant achievement.

Acknowledgments

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X. International Perspectives

New Chapter

Cryopreservation of Fish Sperm and Embryos in China

Song-Lin Chen, Xiang-Shan Ji and Yong-Sheng Tian

Introduction

Programs to preserve genetic diversity of cultured, rare, and threatened fish species, as well as management of species in natural ecosystems would benefit from gamete and embryo cryopreservation. In addition, cryopreservation has applications in selective breeding for disease resistance and stock improvement and for transfer of animals among hatcheries. Cryopreservation of spermatozoa is a powerful technique for preserving fish germplasm and protecting fish species from extinction (Chen et al. 1992a, Chen et al. 1993, Ohta et al. 2001, Robles et al. 2003). In addition, cryopreservation is a prerequisite for establishing gene banks and can provide a year-round supply of fish semen, to support breeding and genetic studies. But variable results are obtained among species and between samples from the same male. Numerous experiments are needed to cryopreserve fish spermatozoa successfully. Most studies on gamete preservation are conducted on a laboratory scale. Not much practical progress of fish spermatozoa cryopreservation in large volume cryovials for the construction of cryobanks or commercial purposes has yet been achieved (Wheeler and Thorgaard 1991, Cabrita et al. 2001, Lahnsteiner et al. 2002, Chen et al. 2004).

Cryopreservation of embryos would be beneficial for conservation of germplasm and genetic improvement of fish. Viable eggs and embryos 8 to 14% dimethyl sulfoxide (DMSO) when eggs were frozen at –20 °C and embryos at –55 °C (Zell 1978, Erdahl and Graham 1980). Recent studies have examined chilling injuries, cooling rate and cryoprotectant toxicity at various stages of embryonic development in different fish species (Robertson et al. 1988, Zhang et al. 1993, Suzuki et al. 1995). Using magnetic resonance and electron spin resonance microscopy, it was established that cryoprotectants were able to permeate zebrafish embryos (Hagedorn et al. 1996, 1997). There is a report of common carp *Cyprinus carpio* embryos surviving cryopreservation by use of a programmed freezing method in liquid nitrogen (Zhang et al. 1989), but results have not been reproducible. Fish embryos are sensitive to conventional cryopreservation procedures because of contravening conditions required to minimize mortality from intracellular ice formation and osmotic injury. Because of their large size (usually 1-7 mm), low cell permeability, presence of two membranes, sensitivity to chilling injury and high yolk content in the egg, cryopreservation of fish embryos is more difficult than cryopreservation of fish sperm and mammalian embryos (Harvey et al. 1983, Gwo 2000). In the present review, we summarize recent progress in cryopreservation of fish sperm and embryos in China.

Chen, S.-L., X.-S. Ji and Y.-S. Tian. 2011. Cryopreservation of Fish Sperm and Embryos in China. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 835-851.

Methods for Cryopreservation of Sperm

Gamete Collection

Semen was collected from fishes by syringe after applying gentle abdominal pressure and was transferred into 25-mL bottles. The semen concentration was determined using the equation $SC = (0.806\ OD - 0.032) \times 10^8$, where "SC" is semen concentration (cells/mL) and "OD" is optical density at a 260-nm wavelength (Fauvel et al. 1998). Eggs were collected by abdominal pressure of females and placed in a 500-mL beaker. About 5 mL of eggs were added to a 250-mL beaker containing 200 mL of fresh seawater to test egg quality. If the majority of the eggs floated, this batch of eggs was used in experiments.

Motility Assessment

Sperm were activated by adding a drop of seawater to the sperm spread on a glass slide. After activation, sperm motility was immediately determined by use of light microscope at 100-x magnification. The motility was expressed by values from 0 to 5, with "0" representing no motile spermatozoa, and "5" from 80% to 100% of sperm showing progressive movement. To validate the motility measurement, preliminary tests were carried out using three samples.

Freezing Methods

Semen was cryopreserved using the "three-step" method (Chen et al. 1992a,b). In brief, within 10 min of collection, semen was mixed with pre-cooled (4 °C) extenders containing cryoprotectants at a ratio of 1:1 (extender:semen) and was equilibrated at 4 °C for 30 min. The equilibrated semen was transferred to 1.8-mL cryovials (Nalgene Inc., Rochester, New York, USA). The filled cryovials were transferred into a gauze pocket 6 cm wide and 9 cm long and were held for 10 min in liquid nitrogen vapor at 2 cm, 6 cm, or 13 cm above the liquid nitrogen surface, lowered and held for 5 min on the surface of liquid nitrogen, and finally immersed in liquid nitrogen. The cooling rate of semen in the cryovial was measured using a programmable freezer (Shanghai University of Science and Technology, Shanghai, China). The effect of cooling rate on the motility of thawed semen was also examined.

There were three extenders used in these studies. Modified plaice Ringer solution (MPRS) was modified according to Yao et al. (2000). Extender D-15 was prepared as described previously (Chen et al. 1992a). In addition, MMM (modified Mounib medium) was also investigated (Dreanno et al. 1997). The effects of type and concentration of cryoprotectant on motility of post-thaw semen were determined using DMSO, methanol (MEOH), dimethl formamide (DMF) ethylene glycol (EG) propylene glycol (PG) and glycerol (Amresco Inc., Solon, Ohio, USA) as cryoprotectants.

Thawing Methods

After storage for 1 d to 1 yr in liquid nitrogen, the gauze pocket containing the cryovials was equilibrated for 5 min in liquid nitrogen vapor and removed from the liquid nitrogen. Semen was thawed in a water bath at 37 °C: the thawing time for 0.5 mL of semen was 50 sec; for 1 mL was 1.5 min, and 1.8 mL was 2.5 min. The motility of the thawed sperm was determined as

described above.

Morphology Assessment

First, the semen was cryopreserved using MPRS supplemented with 10% DMSO. The volume of diluted semen in the cryovial was 1 mL. The frozen semen was thawed in a water bath at 37 °C. Fresh and thawed semen were fixed in 3% glutaraldehyde in seawater. After 15 min, the supernatant was removed and the pellet was resuspended in fresh 3% glutaraldehyde in seawater. For scanning electron microscopy, the samples were fixed in 1% osmium tetroxide, dehydrated with a graded ethanol series and dried on dry ice. Observation was carried out using a scanning electron microscope (JEOL, JOM 7000). For transmission electron microscopy, the samples were dehydrated with a graded ethanol series, embedded in Epon812 media (Sigma) and sectioned with a LKB slicer (Stockholm, Sweden).

Fertilization Trials

Fertilization trials were conducted to compare the fertility and hatching of fresh and thawed semen. Generally, fresh eggs were inseminated with different volumes of thawed sperm. After mixing of the gametes, a small quantity of seawater or fresh water (about 100-200 ml) was added to the mixture of semen and eggs to activate the gametes. The eggs were incubated at different temperatures (varying from 11 to 23 ^{o}C according to fish species) and dead eggs were removed in a timely fashion. When the eggs developed to gastrula stage, the fertilization (number of gastrula stage embryos/number of eggs) was determined. The hatching was expressed as the percentage of hatched larvae in the fertilized eggs. The eggs from one female were used for each experiment, and each experiment was replicated 3 times with semen from the same male.

Statistical Analysis

Data were expressed as the mean ± SEM. Motility after activation was analyzed using one-way analysis of variance (ANOVA). When differences were significant, the LSD (Least Significant Difference) test and Tukey test were used for comparisons. A value of $P < 0.05$ was considered to be statistically significant. Fertilization and hatching were tested by independent-samples T-test. Statistical analyses were computed using SPSS software.

Sperm Cryopreservation of Chinese Carps

During 1985-1992, cryopreservation of spermatozoa was carried out for four species of Chinese carps: grass carp *Ctenopharyngodon idella*, silver carp *Hypophthalmichthys molitrix*, common carp *Cyprinus carpio* and blunt snout bream *Megalobrama amblycephala* (Chen et al. 1992a,b). A series of experiments was carried out to develop repeatable and straightforward techniques. Effects of the composition of diluents on sperm motility before freezing and after thawing were studied, and slightly acidic diluents containing NaCl, KCl and glucose yielded higher post-thaw motility and fertility than did slightly basic diluents. Among the NaCl/KCl/glucose diluents, D-15 gave the best results for grass carp and silver carp, while D-16 and D-17 appeared to be more appropriate for the sperm of common carp and blunt snout bream.

Cryoprotection by DMSO was examined and freezing of sperm resulted in a marked increase of glutamate-oxaloacetate transaminase enzyme activity and other protein levels in seminal plasma. The addition of DMSO to the diluted sperm mixture before freezing significantly reduced the effect of freezing damage by increasing the osmotic pressure and lowering the freezing point. The effects of DMSO concentrations on the motility and duration of post-thaw sperm were observed: DMSO at 6-18% always provided cryoprotection; 10-12% DMSO gave the highest post-thaw motility, and 8% DMSO gave the longest duration of motility. Comparison of the effects of different freezing containers on sperm survival and fertility indicated that plastic tubes were superior to glass ampules for cryogenic storage of carp sperm. The effect of osmotic pressure of activator solution on sperm survival was studied. Solutions with osmotic pressures of 256-510 mOsmol/L were most suitable for the reactivation of frozen sperm and solutions with 170-256 mOsmol/L gave longer duration of motility. Fertility of thawed sperm was higher when ova were inseminated in the solution above than in fresh water. The efficiency of the cryopreserved sperm could only be estimated when the sperm-to-ova ratio was known. The lowest density of frozen sperm required to obtain satisfactory fertilization (over 80%) was determined to be 5×10^5 sperm/egg. These studies demonstrated that the sperm of Chinese carps could be stored in liquid nitrogen for 2 d to 1 yr with high post-thaw motility (60-70%) when sperm were preserved in D-15, D-16, or D-17 diluents. Frozen sperm could fertilize ova almost as effectively as fresh sperm, and the fertilization was between 80-94%. The hatching from thawed sperm was the same as the control group (Chen et al. 1992a,b, 1993).

Sperm Cryopreservation of Turbot

Studies have been performed on cryopreservation of sperm from turbot *Scophthalmus maximus* (Chen et al. 2004). The effects of various extenders, cryoprotectants and sperm-egg insemination ratios on motility fertilization capacity of thawed sperm were examined to optimize cryopreservation procedures. Post-thaw motility obtained with extender TS-2 was higher than that achieved with extenders D-15 and MPRS. The most effective cryoprotectant was determined to be 10% DMSO. Fertilization of small egg batches (2 mL) with thawed sperm resulted in a fertilization of 70 ± 9% and hatching of 47 ± 5% similar to the fertilization (75 ± 8%) and hatching (48 ± 7%) of fresh sperm. The minimal density of frozen sperm required to obtain satisfactory fertilization was determined to be 2000:1 (sperm:egg). Fertilization of larger batches of eggs (40 mL) with sperm frozen in 1.8-mL cryovials provided similar fertilization (72 ± 7%) to that of the fresh sperm (76 ± 6%), whereas the hatching (35 ± 4%) of eggs fertilized with frozen sperm was lower than that of fresh sperm (41 ± 4%). Our results demonstrated that cryopreservation of turbot sperm in 1.8-mL cryovials could be used for hatchery purposes.

Sperm Cryopreservation of Sea Perch

A method was developed for cryopreserving sperm of sea perch *Lateolabrax japonicas* in 1.8-mL cryovials (Ji et al. 2004). In brief, the effects of various extenders, cryoprotectants, and volumes of diluted semen on the motility of post-thaw spermatozoa were examined. Post-thaw

motility obtained with extender MPRS was higher than that for extenders D-15 and MMM. With MPRS, the most effective cryoprotectant was 10% DMSO. Post-thaw motility was not significantly reduced when the volume of diluted semen in the cryovial was increased from 0.5 mL to 1.0 mL ($P > 0.05$). When the sperm:egg ratios varied from 320,000:1 to 20,000:1, fertilization of semen held for 3 d or 1 yr in liquid nitrogen were not significantly different from that of fresh sperm ($P > 0.05$). In fertilization trials of 230 mL of eggs with thawed semen held for 3 d in liquid nitrogen, 85% fertilization and 70% hatching were obtained, which was similar to controls (81 ± 2% and 87 ± 3%) ($P > 0.05$). Insemination of large egg batches (440 mL) with frozen sperm held for 1 yr in liquid nitrogen resulted in high fertilization (84%) and hatch (90%) comparable to these obtained with fresh (control) semen ($P > 0.05$). Scanning and transmission electron microscopic observation indicated that while most of thawed sperm remained morphologically normal, some exhibited damage, which probably caused the decrease in motility and fertility of the thawed sperm (Ji et al. 2004).

Sperm Cryopreservation of Spotted Halibut

Studies have been performed on cryopreservation of sperm from spotted halibut *Verasper variegatus* (Tian et al. 2008). The effects of various extenders and cryoprotectants on motility of thawed sperm were examined. The motility of thawed sperm in TS-2 was higher than that in ASW and MPRS ($P < 0.05$) and was not significantly different from that of fresh sperm ($P > 0.05$). While the motility of sperm cryopreserved with 13.3% EG, 13.3% glycerol, 13.3% MeOH and 13.3% DMF was less than 5%, no significant differences were observed in the motility between fresh sperm and thawed sperm cryopreserved with 13.3% DMSO, 13.3% PG ($P > 0.05$). Using the above method, spotted halibut semen was cryopreserved with extender TS-2 and 13.3% DMSO or 13.3% PG. As a result, the fertilization (35 ± 11%) and hatching (24 ± 12%) from thawed sperm were not significantly different from that of fresh sperm ($P > 0.05$). The motility and activation time of thawed sperm activated by artificial seawater at different salinities were different. Low salinity (osmolality) delayed the activation of thawed sperm. The highest motility was observed with 3% artificial seawater ($P < 0.05$). The most suitable temperature of seawater to activate spotted halibut sperm after thawing was 18 °C ($P < 0.05$). However, temperature of seawater had no significant effect on time delay ($P > 0.05$).

Sperm Cryopreservation of Stone Flounder

Sperm cryopreservation was investigated in stone flounder *Kareius bicoloratus* (Ji et al. 2005). The extender MPRS was selected and motility of thawed sperm was more than 70%. To confirm that the thawed sperm from stone flounder had fertilization ability with eggs from Japanese flounder *Paralichthys olivaceous* for hybridization (Japanese flounder ♀ × stone flounder ♂) breeding trials were performed. The hybrid embryos hatched 70-75 hr after fertilization. No significant difference was found between hybridized group and control (Japanese flounder ♀ × Japanese flounder ♂). Hybridization trials between turbot eggs and Japanese flounder frozen sperm was also performed, but all the hatched hybrid fry were abnormal because of the chromosome differences.

Sperm Cryopreservation of Olive Flounder

Sperm cryopreservation was studied in olive flounder *Paralichthys olivaceus* (Zhang et al. 2003). Three cryoprotectants were studied. Post-thaw motility was 61 ± 4% and fertilization was 67 ± 15% for DMSO, motility was 79 ± 5% and fertilization 76 ± 10% for glycerol, and motility was 13 ± 5% and fertilization was 45 ± 23% for MeOH. The hatching of fertilized eggs was 37 ± 8% for DMSO, 48 ± 26% for glycerol, and 23 ± 11% for MeOH. When viewed by scanning electron microscopy, the majority of the thawed sperm remained morphologically normal, some exhibited lost or dilated mitochondria, swollen mid-pieces, broken tails, or damaged cell membranes (Zhang et al. 2003).

Sperm Cryopreservation of Red Sea Bream

The physiological characteristics of red sea bream *Pagrus major* spermatozoa at various salinities and pH were investigated along with cryopreservation (Hong et al. 1996). Post-thaw motility and swimming time of thawed sperm were used as criteria to evaluate cryopreservation. The results indicated that the extender composed of 0.8% NaCl and 0.5% KCl were appropriate. Potassium was necessary for increasing the survival rate and glycine could prolong swimming time of thawed sperm. Although glycerol, DMSO and MeOH were appropriate cryoprotectants, the mixture of 8% glycerol and 4% DMSO was the most effective. Freezing of semen yielded better results than freezing of testes. Fresh spermatozoa gave the highest average survival rate and the longest swimming time at pH of 8.08 and salinity of 30 ppt (Hong et al. 1996).

The cryopreservation protocol for this species was optimized recently (Liu et al. 2007) with use of a programmable freezer and 2-mL cryovials. Four cooling rates and three thawing temperatures were tested. The best post-thaw motility (79 ± 5% to 89 ± 8%), fertilization (90 ± 3% to 96 ± 2%), and hatch (85 ± 5% to 91 ± 4%) were achieved when Cortland extender, DMSO (15, 18, and 20%) or ethylene glycol (9 and 12%) were used for cryopreservation media with 20 °C/min as the cooling rate, and 40 °C as the thawing temperature.

Possibility of Refreezing Thawed Sperm

A method of freezing and refreezing sperm from multi-scaled sharp-jaw barbel *Varicorhinus macrolepis* (sometimes listed as *Onychostoma macrolepis*) (Romero et al. 2009) was developed for use in 1.8-mL cryovials (Ji et al. 2008). The post-thaw motility in extender D-15 was higher than in MPRS and fish Ringer solution ($P < 0.05$). Dimethyl sulfoxide and glycerol provided a higher level of protection than MeOH during cryopreservation. The most effective concentration of DMSO and glycerol was determined to be 10%. Fertilization by thawed sperm was not significantly different from that of fresh sperm. Hatching was not significantly different between thawed sperm (83 ± 12%) and fresh sperm (91 ± 4%). When thawed sperm was refrozen immediately after thawing, the post-thaw motility was 0%. When thawed semen was refrozen after diluting with D-15 (containing DMSO) at a ratio of 1:2, the

post-thaw motility of refrozen sperm was 38 ± 3%. Post-thaw motility was lower for refrozen than for once-frozen sperm ($P < 0.05$). The fertilization using refrozen sperm was 43 ± 7% and hatching was 34 ± 11%, which was lower than that of fresh sperm ($P < 0.05$).

Genetic Structure of Offspring Produced with Frozen Sperm

The effect of cryopreservation on sperm nuclear DNA stability of red sea bream was analyzed by comet assay (single cell gel electrophoresis, SCGE), a sensitive method to analyze induction and repair of DNA strand breaks, and oxidative DNA lesions (Xu et al. 2005). Alkali-induced DNA strand damage was divided into 5 grades: no damage (G0), slight damage (G1), mild damage (G2), heavy damage (G3), and total damage (G4). Most sperm with DNA damage were in the grades G1 and G2, and few were totally damaged. The measured comet rate in 5% DMSO was 33 ± 9%; in 10% DMSO was 36 ± 19%; in 18% was 49 ± 9%; in 20% was 43 ± 11%; in 25% was 56 ± 39%; and in 30% was 82 ± 19%. The comet rate of the fresh sperm was 31 ± 3%. Comparison of damage indicated that frozen sperm DNA in 30% DMSO was significantly different from fresh sperm (Xu et al. 2005).

The genetic structure of offspring derived from cryopreserved sperm was studied (Chen et al. 2004). Simple sequence repeats (SSR) was used to analyze the genetic structure of turbot fry from fresh and frozen sperm. Turbot sperm were diluted in TS-2 (Chen et al. 2004) at a ratio of 1:1 (v/v) and cryopreserved by the three-step method. Motility of thawed sperm was 65% and was not significantly different from that of fresh sperm (85%). Two fertilization trials were conducted. Experiment I: After turbot eggs (about 100 mL) from a female and sperm (about 1 mL) from a male were collected, 40 mL of eggs were immediately inseminated with 0.5 mL of fresh sperm. Experiment II: After 0.5 hr of storage in liquid nitrogen of cryopreserved sperm from the same male, frozen sperm (0.5 mL) were thawed and placed on turbot eggs from the same female. In this way, turbot fry were produced with frozen and fresh sperm (15 individuals from each parent were used to decrease the effect of parental genetic type on the results). A total of 30 alleles and 25 genotypes at 10 microsatellite DNA loci were acquired from 15 turbot fry produced from frozen sperm, and the results were the same as fry produced from fresh sperm. The heterozygosity (H) was calculated based on allele frequencies of each microsatellites locus. The total heterozygosity of turbot fry produced from fresh sperm was 0.575, while that of turbot fry produced from frozen sperm was 0.571. Of 30 alleles, only 7 allele frequencies varied significantly.

Vitrification of Embryos from Marine Fish

Experimental Approach

Experiments were conducted, first to determine the relative toxicity to embryos of six cryoprotectants; second to compare toxicity of vitrifying solutions containing the two least toxic cryoprotectants in variable proportions; third to assess tolerance to vitrification of embryos at different development stages; fourth to evaluate methods to equilibrate embryos in vitrifying solutions, and finally to develop vitrification procedures for cryopreservation of flounder embryos. All chemicals used were of high purity: NaCl, KCl, $CaCl_2$, $MgCl_2$ and $NaHCO_3$ were

from Sangon Ltd. (Shanghai, China); PG, MeOH, DMF, EG and glycerol were purchased from Sigma Chemical Corp. (St. Louis, Missouri, USA).

Fish Culture and Embryo Collection

Flounder broodstock were cultured and managed at the Haiyang High-Tech Experimental Station of the Yellow Sea Fisheries Research Institute in Haiyang, China. Before use, 30 females and 10 males of 5-yr-old broodstock were placed in the same tank and held at a constant photoperiod of 16 h of light:8h of darkness and a water temperature of 14-16 °C for 60 d to stimulate maturation and natural spawning. Fertilized eggs and embryos produced from mass spawning in the tanks were collected daily using a small net and were disinfected with 2 ppm of $KMnO_4$. Eggs and embryos were incubated in a 1000-L aerated hatching tank with circulating sea water (salinity, 3.0-3.2%; osmolarity, 917 ± 6 mOsmol/L) at 13-16 °C. Egg density during hatching was 500-1000 eggs/L. Embryo developmental stage was determined as described by Tian et al. (2004). Generally, clear and luminous embryos with a diameter of 0.85-1.0 mm floating on the seawater surface were considered to be of satisfactory quality for use in the cryopreservation trials. Survival of treated embryos was assessed 8-10 hr after treatment. Deformation of fry was assessed by looking for abnormal curvature of the spine.

Handling of Eggs and Embryos

For equilibration in vitrifying solutions, embryos were transferred to 5-cm Petri dishes containing 2 mL of equilibration extender. After each equilibration, 90-95% of the extender was removed using a plastic dropper, and 3 mL of new extender was added for the next equilibration step. Hatched larvae were handled using a large-mouth plastic dropper.

Preparation of Basic and Washing Solutions

Artificial seawater served as starting media for extenders. Preliminary experiments demonstrated that basic saline solution BS2 resulted in the highest survival of embryos. It was used for preparation of all vitrifying solutions and consisted of: 0.423 M NaCl, 0.012 M KCl, 0.01 M $CaCl_2 \cdot 2H_2O$, 0.024 M $MgCl_2 \cdot 6H_2O$ and 0.002 M $NaHCO_3$, at pH 7.63. The osmolarity was 883 ± 3 mOsm/L as determined by vapor pressure osmometer (Gene Company, Hong Kong). As washing solution, 0.125 M sucrose in BS2 was used (osmolarity 1023 ± 4 mOsmol/L, pH 7.74). It resulted in higher survival rates than other sucrose solutions in preliminary experiments.

Assessment of Cryoprotectant Toxicity

Six cryoprotectants *(*PG, MeOH, DMSO, DMF, EG and glycerol) were evaluated for toxicity to flounder embryos. Twenty percent solutions (v/v) were prepared by diluting each cryoprotectant in BS2. The Molar concentration equivalents were 2.74 M for PG, 4.94 M for MeOH, 3.89 M for DMF, 2.79 M for DMSO, 2.71 M for glycerol, and 3.58 M for EG. Because turbot embryos between the 4-somite and 20-somite stages are more resistant to cryoprotectant toxicity than at other developmental stages, these stages were used for toxicity experiments. The embryos were exposed to extenders containing the full concentration of cryoprotectant for 20 min at 13-16 °C, placed in washing solution for 10 min and incubated in seawater. After 10 hr of incubation, viable embryos were counted and survival rates were calculated. The experiment was

repeated 5 times using ~40 embryos in each replicate.

Selection and Preparation of Vitrifying Solutions

The tests above demonstrated that PG and MeOH were the least toxic to flounder embryos and were selected for further studies. Previous study had demonstrated that 3 parts PG to 2 parts MeOH (v/v) resulted in satisfactory vitrification of sea perch embryos. Four vitrifying solutions (FVS1-FVS4) were prepared by combining PG, MeOH and BS2 in different proportions. Flounder embryos at the muscle contraction stage were used to test the vitrifying solutions. Embryos were stepwise equilibrated in 25%, 33%, 50%, 67% and 100% of the vitrifying solutions for 8 min in each step at 13-16 °C, placed in washing solution for 10 min, and transferred into seawater for incubation until hatching. Survival rate (number of live embryos/number of treated embryos), hatching (number of hatched larvae/number of live embryos) and deformation rate (DR: number of deformed larvae/number of hatched larvae) were determined for each vitrifying solution. The experiment was repeated 3 times, and each replicate utilized ~30 embryos.

Vitrification Tolerance of Embryos at Different Developmental Stages

Embryos at the mid-gastrula (25-hr post fertilization (PF), blastopore closure (29-hr PF), 4-5 somite (35-hr PF), 15-20 somite (40-hr PF), tail bud (49-hr PF) and heart beating (67-hr PF) stages were treated in FVS2 in a 5-step manner for 40, 50 or 60 min at 13-16 °C. In brief, the embryos were serially equilibrated in 25%, 33%, 50%, 67% and 100% FVS2 for 8 min, 10 min or 12 min in each step. After equilibration, the embryos were placed in washing solution for 10 min and incubated in sea water for 8-10 hr, before survival was assessed. The experiment was repeated 5 times with ~30 embryos per replicate.

Comparison of Equilibration Methods

Five equilibration protocols were evaluated to identify conditions that would minimize osmotic stress and toxicity of cryoprotectants. Embryos in the tail bud stage were equilibrated in a stepwise manner in FVS2 at 13-16 °C as follows. 1) One-step method: embryos were placed directly in 100% FVS2 for 30 min. 2) Two-step method: embryos were equilibrated in 50% and 100% FVS2 for 15 min in each step. 3) Three-step method: embryos were serially equilibrated in 25%, 50% and 100% FVS2 for 10 min in each step. 4) Four-step method: embryos were serially equilibrated in 25%, 50%, 67% and 100% FVS2 for 7.5 min in each step. 5) Five-step method: embryos were serially equilibrated in 25%, 33%, 50%, 67% and 100% FVS2 for 6 min in each step. After equilibration, the cryoprotectant solutions were discarded, and the embryos were placed in washing solution for 10 min, incubated in seawater for 10 h, after which survival of the embryos was determined. Equilibration methods were evaluated based upon survival of embryos exposed to FVS2 for 40-60 min. The experiment was repeated 5 times using ~30 embryos per replicate.

Equilibration and Freezing of Embryos

For vitrification, embryos at the neurula (31-hr PF), 14-somite (38-hr PF), tail bud (49-hr PF), heart beat (67-hr PF), pre-hatching (87-hr PF) and hatching stages (93-h PF) (20-60 embryos per treatment) were used in combination with the four vitrifying solutions. In total, 44 experiments were performed. Embryos at the neurula and hatching stages were vitrified only in FVS2 and FVS3, and these experiments were repeated twice. Embryos at the 14-somite, heart beat and pre-hatching stages were cryopreserved in all four solutions, with each experiment repeated twice. Embryos at the tail bud stage were cryopreserved in all four solutions, and that with each experiment repeated 3 times. In all experiments, equilibration of embryos was performed in five steps at 13-16 °C.

Following osmotic equilibration in FVS, 9-12 embryos in 250 uL of vitrification solution were drawn into 0.5-mL plastic straws. The straws were sealed by heating and were plunged directly into liquid nitrogen (-196 °C) in a 15-L dewar (Yaxi Co. Ltd., China) for cryopreservation. Vitrification was assessed by visual inspection. Ice formation in straws resulted in whitening of the solution, while straws remaining transparent (generally about 70% of those attempted) were considered to be vitrified.

Thawing and Incubation of Embryos

After storage for 1-7 hr in liquid nitrogen, straws containing frozen embryos were immersed in a water bath at 40 °C for rapid thawing (about 7 sec). After thawing, the two ends of the straw were cut, and embryos were placed in 2 mL of washing solution for 10 min. About 8 mL of filtered seawater at 13-16 °C was slowly added to dilute the thawed embryos, and after 10 min the thawed embryos were transferred to about 0.5 L of fresh filtered seawater for incubation at 13-16 °C until hatching or death. Numbers of viable embryos were determined 5-8 h after thawing.

Embryo Vitrification of Olive Flounder

The toxicity and cryoprotective effect of five commonly used cryoprotectants DMSO, glycerol, MeOH, PG and EG were tested on olive flounder embryos (Zhang et al. 2005). Five concentrations of each of the cryoprotectants and 20 different combinations of these cryoprotectants were tested. The toxicity to embryos were in the following sequence: PG < MeOH < DMSO < glycerol < EG ($P < 0.05$); whereas the protective efficiency of each cryoprotectant at -15 °C for 60 min, were in the following sequence: PG > DMSO, MeOH, glycerol > EG (for all greater symbols, $P < 0.05$; and the rest, $P > 0.05$). Methanol combined with any one of the other cryoprotectants gave the best protection. Toxicity was concentration-dependent with the lowest concentration being the least toxic for all five cryoprotectants at 16 °C. For PG, MeOH, and glycerol, 20% solutions gave the best protection at -15 °C; whereas a 15% solution of DMSO, and a 10% solution of EG, gave the best protection at -15 °C (Zhang et al. 2005).

Embryo Vitrification of Japanese Flounder

Vitrification of Japanese flounder embryos was investigated (Tian et al. 2005). The survival of embryos decreased gradually with the prolongation of treatment time, and best survival in VS2 in extender BS2 was observed for the 4-5 paired somites, 16-20 paired somites and tail-bud stages. The type and concentration of washing solutions were evaluated and survival rate was highest when the 0.125mol/L sucrose was used for washing. Survival of 15-20 paired somites was higher at 21-22 °C than at 6 °C and the survival of other stages was opposite (Tian et al. 2005).

An updated vitrification procedure for Japanese flounder embryos was developed (Chen and Tian 2005). In initial toxicity tests, PG and MeOH were less toxic than DMF or DMSO, whereas EG and glycerol were toxic to all tested embryos. Four vitrifying solutions (FVS1-FVS4) were prepared by combining BS2 and the cryoprotectants PG and MeOH in different proportions (FVS1: 67, 20 and 13%; FVS2: 60, 24 and 16%: FVS3: 55, 27 and 18%; FVS4: 50, 30 and 20% of BS2, PG and MeOH). FVS1 produced the highest survival; whereas the deformation rate was highest for FVS4. Five-step equilibration of embryos in FVS2 resulted in higher survival than equilibration in 4, 3, 2 or 1 steps. Embryos varying from the 14-somite to the pre-hatching stages were cryopreserved in the four vitrifying solutions in liquid nitrogen for 1-7 h. From eight experiments, 20 viable thawed embryos were recovered from 292 cryopreserved embryos. Fourteen larvae with normal morphology hatched from the 20 surviving thawed embryos from five experiments. Embryos at the tail bud stage exhibited greater tolerance to vitrification than embryos at other stages. These results established that cryopreservation of flounder embryos by vitrification is possible (Chen and Tian 2005).

The vitrification conditions of Japanese flounder embryos were further optimized (Zhao et al. 2005). Several factors (vitrification solutions, straw diameter, embryo stage, equilibration time, equilibration temperature, wash solution concentration and washing time) were studied. The solution PMDD 2% PVP containing cryoprotectants could vitrify and its devitrification rate was also low when 2.5-mm diameter straws were used in experiments, so it was suitable for embryo vitrification. 2) Embryos at tail bud stage were more tolerant to vitrification solutions, which could completely penetrate into Japanese flounder embryos when equilibrated for 40 min. With equilibration times exceeding 40 min, the survival of embryos decreased significantly ($P < 0.05$). Embryos at every stage were sensitive to equilibration temperature. The survival at 15 °C was significantly lower than at 0 °C and 4 °C ($P < 0.05$). 3) When washing concentrations and time varied, the survival rate of embryos did not significantly change ($P > 0.05$). 4) Based on the optimized conditions, vitrification experiments of Japanese flounder embryos were conducted. From four experiments, 8 viable embryos were obtained from 26 cryopreserved embryos and 7 larvae with normal morphology hatched. The survival time of 7 larvae varied from 2 to 13 d and the hatching varied from 12% to 60% (Zhao et al. 2005).

Embryo Vitrification of Red Sea Bream

A vitrification protocol was developed for red sea bream embryos (Ding et al. 2007). Their method was as following: The toxicity of five single-agent cryoprotectants, DMSO, PG, EG, glycerol, and MeOH, as well as nine cryoprotectant mixtures (Table 1), were examined by hatching rate. Two vitrifying protocols, a straw method and a solid surface vitrification method (copper floating over liquid nitrogen), were compared with post-thaw embryo morphology. Exposure to any single cryoprotectant at 10% concentration for 15 min was not toxic to embryos. However a longer exposure (30 min) at higher concentrations (20 and 30%) of DMSO and PG were less toxic than the other cryoprotectants. Among nine cryoprotectant mixtures, the combination of 20% DMSO +10% PG+ 10% MeOH had the lowest toxicity. High percentages of morphologically intact embryos (51 ± 17%) were achieved by the straw vitrifying method (20.5% DMSO +15.5% acetamide +10% PG, thawing at 43 °C, and washing in 0.5 M sucrose solution for 5 min) and 78 ± 16% was obtained for the solid surface vitrification method (40% GLY, thawing at 22 °C, and washing in 0.5 M sucrose solution for 5 min). After thawing, morphological changes in the degenerated embryos included shrunken yolks and ruptured chorions. Furthermore, thawed embryos that were morphologically intact did not consistently survive from incubation (Ding et al. 2007).

Table 1. Percent composition of cryoprotectant mixtures used for vitrification of red sea bream embryos (taken from Ding et al. 2007).

Mixture number	PG	DMSO	MeOH	ACE	PEG	DMF	EG
1	10	20.5	--	15.5	6	--	--
2	30	--	20	--	--	--	--
3	23	23	--	--	--	--	--
4	10	10	10	--	--	--	--
5	--	37	--	--	--	18.5	--
6	10	30	--	--	--	--	--
7	20	--	20	--	--	--	--
8	10	20	10	--	--	--	--
9	20	--	--	--	--	--	20

Abbreviations: propylene glycol (PG), dimethyl sulfoxide (DMSO), methyl alcohol (MeOH), acetamide (ACE), polyethylene glycol (PEG), dimethyl formamide (DMF), and ethylene glycol (EG).

Other studies obtained morphologically intact embryos of 5-51% with the straw vitrification method and 11-78% with solid surface vitrification method (Li et al. 2007a). Among nine cryoprotectant mixtures, the combination of 20.5% DMSO plus 15.5% acetamide plus 10% PG plus 6% PEG resulted in the highest morphological integrity and the combination of DMSO plus DMA (2 M:1 M) also yielded high morphological integrity. In addtion, use of the single-agent cryoprotectant 40% glycerol, with the solid surface vitrification method, yielded the highest morphologically intact embryos after thawing. In total, 25 red sea bream embryos

survived the vitrification experiments, and some survived for 13 d after thawing (Li et al. 2007a).

Embryo Vitrification of Sea Perch

Vitrification of sea perch embryos was studied by Tian et al. (2003). Five vitrification solutions were selected. The cryoprotectant concentrations in the five vitrification solutions were low, to reduce the toxicity. The vitrification rate of the five vitrification solutions was 48-100% during freezing, and 44-63% during thawing in a 35-43 °C water bath. The solution VSD2 was more stable during thawing than other four vitrification solutions and was used to cryopreserve embryos in the experiment. The exposure time of neurula, 20-pair-muscle, tail-bud, heartbeat and prehatching stages in VSD2 were compared. The neurula stage sustained the least time in VSD2 and the heartbeat stage could endure the longer time. Thus the heartbeat stage selected for vitrification. In addition, the pre-hatching stage was also feasible for vitrification. The effect of different washing times in 0.5 M sucrose was compared and washing for 10-20 min was found to be efficient. Use of vitrification solution VSD2 at -196 °C for embryos at different stages yielded 2-28% transparent embryos after thawing. Two thawed embryos at heartbeat stage survived 42-50 hr after thawing. Another thawed embryo at pre-hatching stage hatched.

Embryo Vitrification of Turbot

The suitability of vitrification solutions in cryopreservation of turbot embryos was examined by Tian et al. (2005). Among several solutions, PMP1 gave highest survival (76%) and was considered to be most suitable. The vitrification solutions with PG and MeOH in the proportion of 3:2 with over 41% cryoprotectant concentration did not freeze and the freezing points of 33-40% cryprotectants were between -32 and -65 °C. The freezing point decreased with the increase of cryoprotectant concentration. Turbot embryos from 4-5 paired somites to tail bud storages were more resistant to cryoprotectants. After vitrification and storage in liquid nitrogen for 14 hr and a live embryo hatched.

Programmed Freezing of Embryos from Japanese Flounder

A programmed method for cryopreservation of embryos was developed to preserve genetic resources of Japanese flounder (Wang et al. 2008). In toxicity tests, flounder embryos were exposed to five cryoprotectants (20% PM (PG + MeOH), 20% FP (DMF + PG), 20% FM (DMF + MeOH), 20% SP (DMSO + PG), and 20% SM (DMSO + MeOH)) for 50 min or 120 min. Results showed 20% PM was less toxic to embryos than others. The optimal concentration of PM was 20-25%. During seeding procedure, temperature reached super-cooling. Especially, when seeded at -12 °C, the temperature was harmless to embryos. The cooling rate was -2 °C/min before seeding, and -8 °C/min rate after seeding. The freezing finished at -45 °C followed by plunging into liquid Nitrogen. In the freezing experiment, 5 embryos were recovered successfully and survived 2-7 d after thawing.

Programmed Freezing of Embryos from Red Sea Bream

The cryopreservation of red sea bream embryos was studied by a programmed freezing method (Li et al. 2007b) by comparing the hatch of embryos immersed in 5% and 15% glycerol for 15 min, the percentage of morphologically intact embryos after cryopreservation, and the time embryos were immersed in extenders. Heartbeat-stage embryos were selected because of better tolerance to cryoprotectant toxicity and low temperatures. Toxicity testing of four single-agent cryoprotectants (DMSO, PG, EG, and glycerol) showed that embryos had best tolerance to PG. The embryos could be immersed in PG for more than 2 hr, but only for 1.5 hr in DMSO, 1 hr in EG, and 1 hr in glycerol. The toxicity immersion time and cryoprotectant concentration were related. Embryos tolerated mixed cryoprotectants better than single agents at the same concentration. Embryo survival was improved when PEG, PVP, and sucrose were added to 8%MeOH at 0-4 °C. Conversely, PEG and PVP increased cryoprotectant toxicity.

In programmed freezing, the survival of embryos frozen to -30 °C was high, especially with 10% PG (81 ± 9% survival). The survival of embryos frozen to -40 to -110 °C was lower than that for freezing to -30 °C. There were 6 surviving embryos (of 253) stored in liquid nitrogen for 22 hr, and 13 surviving (of 123) cryopreserved at -196 °C for 2 hr. Some thawed embryos survived for 10-15 d and then died (Li et al. 2007b).

Programmed Freezing of Embryos from Sea Perch

Programmed freezing methods were studied for sea perch embryos (Yu et al. 2004). Extender DS1 was selected from 8 extenders by 10 hr exposure at the blastopore closure stage. Embryos at the muscular contraction stage tolerated higher concentrations of DMSO than did other stages. Survival of seeded groups was higher than that of non-seeded groups, and the 2-step method (embryos first placed in 0.25 M sucrose for 10 min and then in seawater) was better than the 1-step or 3-step methods. Cryopreservation embryos was done with different cooling rates (0.4 °C/min, 1.0 °C /min, 1.2 °C /min, 1.5 °C /min, 2.0 °C /min) and 3 of 72 embryos at the heartbeat stage survived a cooling rate 1.5 °C/min and being held in liquid nitrogen for 30 min before thawing (Yu et al. 2004).

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New Chapter

Development of a Sperm Cryopreservation Approach to the Fish Biodiversity Crisis in Bangladesh

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Introduction

Currently there are valid scientific descriptions and proper identifications for about 24,600 living species of fishes within 482 families and 57 orders (Nelson 1994). Among the six normally recognized freshwater zoogeographic regions (Helfman et al. 1997), the Oriental Region includes Bangladesh, India, Pakistan, Nepal, Bhutan, Sri Lanka, the other Southeast Asian countries, the Philippines and most of the Indonesia. Without doubt, freshwater biodiversity is facing crisis worldwide, but the severity of this threat in the Oriental Region is more intense than any in other geographical area (Dudgeon 2005).

Bangladesh has a unique position in the sub-tropical region, situated within the deltas of three great rivers - the Ganges, the Brahmaputra and the Meghna - covering a total area of 14.3 million ha. It has 290 rivers of which 54 are international, and numerous ponds, *beels* (relatively large waterbodies with static water in the Ganga-Brahmaputra flood plains of Bangladesh), *haors* (wetlands in the northeastern part of Bangladesh which are a bowl or saucer shaped shallow depressions), *baors* (an oxbow lake, found mostly in moribund deltas as in northeastern Bangladesh), lakes, flood plains, brackish, and marine water bodies. The country is rich in the diversity of fish species and is ranked third in aquatic biodiversity in Asia behind China and India, with approximately 300 freshwater and brackish fish species (Hussain and Mazid 2001). Asia represents about 40% of the world total of species of plants and animals and its aquatic environment is the most diverse in the world (Asian Development Bank 2000).

The vast wetlands, wide river-fed systems, abundant rainfall and warm temperatures play a significant role in the ecosystem diversity of Bangladesh. Enormous freshwater fisheries resources feed millions of people living in the Delta. Bangladesh produces about 2.7 million metric tons of fish per year and about 81% of the total production comes from inland resources of which 42% are from culture and 39% are from capture fisheries (DoF 2010). Increasing human population pressure requires accelerated fish production in the country, yet fish seedstock quality is deteriorating. Negative selection, inbreeding and interspecific hybridization in hatcheries have resulted in poor growth, performance and has become a serious constraint to increasing fish production in Bangladesh (Sarder et al. 2005).

The major threats to freshwater biodiversity in Bangladesh are overexploitation, water removal, pollution, massive destruction or degradation of fish habitat, and introduction and invasion of exotic species. Rapid extraction of fish seedstock (for aquaculture) as well as broodfish (for seed production and consumption) from natural waterbodies combined with destructive and unregulated fishing practices (e.g., use of destructive traps, pesticides, gillnets, and complete dewatering of waterbodies) has threatened a number of valuable native species. Loss of aquatic habitat due to siltation, anthropogenic activities such as dam construction (mainly for flood control, irrigation and drainage), and unregulated construction of polders (natural depressions enclosed by embankments), hydroelectric generation, and construction of road networks have been major causes of freshwater species loss. In addition, freshwater

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resources are subject to severe competition among multiple human stakeholders such as crop farming, aquaculture, and industrial usage. This article aims to describe the present status of freshwater fish biodiversity in Bangladesh and current and emerging conservation strategies including sperm cryopreservation for cultured and wild species.

Status of the Freshwater Fishery

Capture fisheries, which are second only to agriculture in the overall economy of Bangladesh, comprise nearly 5% of the gross domestic product (GDP), 23% of gross agricultural production and 6% of total export earnings. It accounts for about 6% of the total protein intake and about 60% of animal protein intake in the diet of the people of Bangladesh (DoF 2010). The fisheries sector provides full-time employment to an estimated 1.2 million fishermen, and an estimated 10 million households are partly dependent on fishing full or part-time for family subsistence in the floodplain. The total fish production according to the 2008-09 survey was 2.7 million metric tons (DoF 2010) (Table 1).

Table 1. Status of water areas and fish production in Bangladesh in 2008-09 (after DoF 2010).

Resource type	Water area (ha × 1,000)	Production (MT × 1,000)	Percent of total production
Inland Fisheries			
Capture			
1. Rivers and estuaries	853.9	138.2	
2. *Beel*	114.2	79.2	
3. Floodplain	2,832.8	879.5	
4. Kaptai lake	68.8	8.6	
5. Sundarbans	177.7	18.5	
Capture Total	4,047.3	1,124.0	41.6
Culture			
1. Ponds and ditches	305.0	912.2	
2. *Baors*	5.5	5.4	
3. Coastal shrimp farms	218.9	145.6	
Culture total	528.4	1,063.2	39.4
Inland total	4,575.7	2,186.7	81
Marine fisheries	-	514.6	19
Country total	-	2,701.3	100

However in recent years, due to several man-made and natural causes, aquatic biodiversity, especially diversity of fish and other non-fish aquatic organisms in open waters, has been declining sharply. During the 1940s and 1950s fish supplied about 95% of the animal protein requirement of the people in Bangladesh, which dropped to 80% in the 1980s (Karim and Ahsan 1989), and to 60% in recent years (DoF 2010). The number of listed freshwater fish species in Bangladesh is 260 comprising 158 Genera, 52 Families and 13 Orders (Table 2). Many of the listed species, however, are under severe threat and are rarely available in natural waters.

Recent studies have shown that the number of freshwater fishes has been declining at an alarming rate. There are 54 species of freshwater fishes are threatened in Bangladesh, and require immediate measures to protect and conserve them (IUCN-Bangladesh 2000) (Table 3). Among the threatened species, 14 are categorized as vulnerable, 28 as endangered, and 12 as critically endangered; another 66 species are classified as data deficient, and only146 as not threatened. Based on the Red Book of the World Conservation Union (IUCN) and reports from different countries, 56 cyprinid species are listed as threatened in 14 Asian countries. This list is not comprehensive and hence there is the likelihood that far more fish species than those reported are either extinct or threatened (Froese and Torres 1999).

Table 2. Freshwater fish species of Bangladesh grouped in 13 Orders.

Order	Family	Genus	Species
Anguiliformes	5	6	7
Osteoglossiformes	1	2	2
Elopiformes	1	1	1
Clupeiformes	3	12	16
Cypriniformes	4	36	86
Siluriformes	13	36	62
Cyprinodontiformes	1	1	1
Syngnathiformes	1	3	3
Synbranchiformes	2	4	6
Perciformes	14	46	59
Beloniformes	3	5	7
Pleuronectiformes	3	4	7
Tetraodontiformes	1	2	3
Total	52	158	260

Conservation Strategy

As more fish species become threatened or endangered, there is tremendous need to preserve disappearing genetic material as well as to conserve the existing gene pools. The ideal strategy for conservation of threatened and endangered fish species is through *in-situ* protection (i.e., habitat restoration) of the native habitat. Unfortunately, most habitat damages are irrevocable and where remediation is possible it is costly and requires a great deal of time, as the restoration process is slow. One alternative is to maintain *ex-situ* conservation (outside the natural environment) as live populations or in a cryopreserved sperm bank (Pullin et al. 1991). The different initiatives taken at government and non-government levels towards *in-situ* and *ex-situ* conservation of freshwaters fishes are highlighted below.

In-situ Conservation

The inland open water fishery resources of Bangladesh are among the richest in the world. They have contributed more than 90% of the country's fish production in the past. However over the last four decades these resources have experienced, as stated above, significant declines due to man-made and natural causes such as overfishing, destructive fishing, loss and destruction of habitats, poor policy, and a lack of planning and management. Fish stocks,

particularly, broodstocks have been depleted below replaceable levels. As a result, both fish biodiversity and overall production have been severely affected. The government has taken different measures for protection, conservation, and management of fisheries resources for sustainable production. The National Water Policy has recently emphasized reserving wetlands for fish in a reversal of past trends. Of the other measures taken, fish sanctuaries have been considered as an important tool for protection and conservation with a policy shift towards community participation. The restoration of 'fish sanctuaries' (the deeper parts of the floodplains and river channels where fish survive during lean periods, and where they grow and attain maturity for spawning in the next monsoon) is particularly important. Fish sanctuaries provide shelter and dry-season refuge, provide protection from predators and fishermen, act as feeding grounds, and serve as breeding and nursery grounds.

Table 3. Threatened fish species of Bangladesh (after IUCN-Bangladesh 2000).

English name	Scientific name	Status	English name	Scientific name	Status
Grey featherback	*Notopterus notopterus* *	VU	Reticulate loach	*Botia lohachata*	EN
Bengal eel	*Anguilla bengalensis*	VU	Giant river catfish	*Aorichthys seenghala*	EN
Reba	*Labeo ariza*	VU	Stripped catfish	*Batasio tengara*	EN
Ticto barb	*Puntius ticto*	VU	Butter catfish	*Ompok bimaculatus* *	EN
River catfish	*Aorichthys aor*	VU	Pabda catfish	*Ompok pabda* *	EN
Day's mystus	*Mystus cavasius*	VU	Indian butter catfish	*Ompok pabo* *	EN
Gangetic ailia	*Ailia punctata*	VU	Silond catfish	*Silonia silondia*	EN
Grey eel catfish	*Plotosus canius*	VU	Squarhead catfish	*Chaca chaca*	EN
Gangetic mud eel	*Monopterus cuchia*	VU	Wrestling halfbeak	*Dermogenys pusillus*	EN
Glassy prchlet	*Chanda nama*	VU	Deocata pipe fish	*Microphis deokata*	EN
Indian glassy fish	*Pseudambassis ranga*	VU	Spotted scat	*Scatophagus argus*	EN
Gangetic leaffish	*Nandus nandus*	VU	Mud pearch	*Badis badis*	EN
Snakehead	*Channa orientalis*	VU	Frail gourami	*Ctenops nobilis*	EN
Spiny eel	*Macrognathus aculeatus*	VU	Giant snakehead	*Channa marulius*	EN
Featherback	*Chitala chitala**	EN	Zig-zag eel	*Mastacembalus armatus*	EN
Carplet	*Barilius bendelisis*	EN	Boga labeo	*Labeo boga* *	CR
Carplet	*Barilius vagra*	EN	Nandil	*Labeo nandina* *	CR
Bengala barb	*Bengala elanga*	EN	Baitka	*Labeo pangusia* *	CR
Glass barb	*Chela laubuca*	EN	Olive barb	*Puntius sarana* *	CR
Minor carp	*Crossocheilus latius*	EN	Tor masheer	*Tor tor* *	CR
Minor carp	*Labeo bata* *	EN	Rita catfish	*Rita rita*	CR
Black carp	*Labeo calbasu* *	EN	Schilbeid catfish	*Clupisoma garua*	CR
Kuria labio	*Labeo gonius* *	EN	Schilbeid catfish	*Eutropiichthys vacha*	CR
Cotio	*Osteobrama cotio*	EN	Yellow tail catfish	*Pangasius pangasius* *	CR
Trout barb	*Raimas bola*	EN	Gangetic goonch	*Bagarius yarrellii*	CR
Rasbora	*Rasbora rasbora*	EN	Sisor	*Sisor rhabdophorus*	CR
Nectic loach	*Botia dario*	EN	Barca snakehead	*Channa barca*	CR

*Commercial or aquaculture interest; VU, Vulnerable (high risk of extinction in the medium-term); EN, Endangered (very high risk of extinction in the near future); CR, Critically endangered (extremely high risk of extinction in the immediate future).

The Bangladesh government has established a number of fish sanctuaries under different development projects since 1980, with the most intensive efforts in the last decade, and is planning for more fish sanctuaries in the country (Table 4). However, in many cases sanctuaries are not sustainable and there have been little or no monitoring efforts to properly assess management performance, problems, and constraints to ensure sustainability. In addition, Bangladesh has established several training and research institutes working at fish biodiversity and conservation in addition to the scientists and teachers working in more than ten universities

and non-governmental organizations. Among other major activities, establishment of fish passages and fish-friendly structures (FFS), stock enhancement in floodplains, and creation of social awareness are ongoing through public and private initiatives.

Figure 1. A typical fish sanctuary constructed with bamboo poles and water hyacinth in a river. Additional habitat is created at the bottom with bushy tree branches.

Table 4. Fish sanctuaries established in Bangladesh under different projects through 2007.

	Fish Sanctuaries			
Project name	**Number**	**Water area (ha)**	**Area (ha)**	**Beneficiary***
Fourth Fisheries Projects (FFP)	75	39,040	1,361	59,712
Community Based Fisheries Management-2 (CBFM-2)	164	9,359	85	14,618
Patuakhali Barguna Aquaculture Extension Project (PBAEP)	18	545	26	2,118
International Fund for Agricultural Development (IFAD)	18	525	10	1,477
Fisheries Resource Development Project (FRDP)	15	1,434	-	-
Management of Aquatic ecosystem through Community Husbandry (MACH) project	56	-	277	-
Hilsa Sanctuary	4	-	-	-
Joboi beel Project	4	-	-	-
Fish Habitat Restoration Project (FHRP)	3	642	4.50	
Fisheries Development and Management Programme (FDMP)	20	738	-	2,335
Total	377	52,283+	1,764+	80,260+

*The professional and part-time fishers who receive benefits from the sanctuary. This includes catches for subsistence and family consumption from the site of the sanctuary.

Ex-situ Conservation by Sperm Cryopreservation

Recently there has been expanded development of cryogenic sperm banks for fish in Europe and North America. These sperm banks are more cost effective than maintaining live gene banks which require dedicated facilities, labor and high costs. Cryogenic gene banking avoids the risk of genetic contamination and requires little space and minimal facilities. The sperm cryopreservation protocols for different fish species seem variable and species-specific. Although fish are the main protein source in Bangladesh and other countries in the sub-continent, and the fish biodiversity and production from open water are declining, little attention has been paid to cryopreservation of fish sperm. In India, protocols have been developed with varying success only for a few aquacultured and endangered species (Chao et al. 1992, Rana 1995, Ponniah 1998). The trials have mainly concentrated on development of extenders, activation media, dilution rates, activation periods, and sperm-to-egg ratios among species.

In Bangladesh, research on fish sperm cryopreservation was started in early 2004. Like Indian scientists, research in Bangladesh concentrated on basic conditions such as suitable combinations of extenders and cryoprotectants, optimal dilution ratios (milt:diluent), optimal cryoprotectant concentrations, and assessing the fertilization and hatching success for cryopreserved sperm. The studies have focused on aquacultured or commercial species and so far none of the threatened species have been considered (Table 5). The results of the trials are variable. The extenders used for different species are egg-yolk citrate, urea-egg yolk, 0.8% NaCl, 0.9% NaCl, Mounib A and B solutions, modified Kurokura solution, Kurokura-1, Kurokura-2, Alsever's solution, modified fish Ringer, 0.6% NaCl, 0.3 M glucose solution, 0.3 M fructose solution, and 0.3 M and 0.6 M sucrose solutions among others. Cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol, ethanol, methanol, ethylene glycol, and dimethylamine (DMA) have been combined with the extenders. Egg-yolk citrate and DMSO were the most used extenders and cryoprotectants. Different combinations and compositions of extenders have been used to dilute sperm in different ratios to increase motility or viability after thawing. Unfortunately, osmolality and pH of the seminal plasma and medium were not reported in any of these experiments.

A number of cooling protocols have been used for fish sperm cryopreservation (Leung and Jamieson 1991). In Bangladesh, however, in almost all trials, a two-step freezing protocol has been used from ambient temperature (25 C) to -4 °C at a rate of 4 °C per min, and then from -4 °C to -80 °C at a rate of 10 °C per min. One-step freezing was applied only in tilapia, Indian major carp (*L. calbasu*) and olive barb from 20 °C to -80 °C at a rate of 10 °C per min.

Although breeding success could not be adequately addressed in all of these studies, cryogenic protocols have been developed for nine aquacultured species in Bangladesh. The fertilization of eggs with the cryopreserved sperm was successful and the hatching rate varied between 0% and 90%. The fish produced from the eggs fertilized by cryopreserved sperm performed well and there was no significant deviation in growth when compared with fish produced from eggs fertilized by fresh sperm (Sarder et al. 2005).

Freshwater biodiversity is under threat all over the world, however, the intensity of threat in Bangladesh, is exceptional. In addition, commercial and subsistence fish farmers and hatchery owners have long been complaining about inbreeding problems such as retarded growth rate, poor reproductive performance, morphological deformities, increased incidence of disease, and mortality of hatchery-produced seed (Hussain and Mazid 1999). The extensive stocking in natural water bodies of fish seed of genetically poor quality is also a major concern.

Table 5. Cryopreservation research summary of some fish species in Bangladesh.

Species	Best combination*	Maximum (percent)			Reference
		Post-thaw motility	Fertilization	Hatching	
Indian Major carp *Catla catla*	2	83	--	--	Sarder 2004
	3	83			Islam 2004
	1	82			
Indian major carp *Cirrhinus cirrhosus*	1	83	--	--	Sarder 2004
	2	79			Sultana 2004
Indian major carp *Labeo rohita*	1	85	90	90	Rahman 2006
	4	83			Rafiquzzaman 2004
	2	81			
Silver carp	1	75	--	--	Ahmed 2004
Hypophthalmichthys molitrix	6	70			Khan, 2004
	7	60			Hossain 2005
Bighead carp	1	75	40	10	Khan 2004
Aristicthys nobilis	6	70			
	7	60			
Common carp	8	86	--	15	Salam 2005
Cyprinus carpio	9	82			
Silver barb *Barbonymus gonionotus*	1	70	--	27	Hasan-ud-doulha 2004
	2	78			Shafin 2006
	10	74			
Nile tilapia	1	80	20	20	Rafiquzzaman 2004
Oreochromis niloticus	5	70			
Indian major carp *Labeo calbasu*	4	74	60	15	Ongoing
	11	68			
Olive barb	4	69	70	30	Ongoing
Puntius sarana	9	66			
	12	63			
Butter catfish *Ompok bimaculatus*					Ongoing
Zig-zag eel *Mastacembelus armatus*					Ongoing
Striped snakehead *Channa striatus*					Ongoing
River catfish *Rita rita*					Ongoing

*Best combinations in terms of post-thaw motility reported for different species. 1: Egg-yolk citrate with DMSO; 2: Urea-egg yolk with DMSO; 3: Egg-yolk citrate with methanol; 4: Alsever's solution with DMSO; 5: Egg-yolk citrate with methanol; 6: Egg-yolk citrate with DMSO and glycerol; 7: Urea-egg yolk with Glycerol; 8: Alsever's solution with ethanol; 9: Alsever's solution with methanol, and 10: Modified Alsever's solution with methanol, 11. 0.9% NaCl with DMSO, and 12. 0.9% NaCl with glycerol.

Fish sperm cryopreservation assists conservation of fish biodiversity through gene banking of endangered species, and assists aquaculture by providing flexibility in spawning of females and selective breeding through synchronizing artificial reproduction, efficient utilization of semen, and maintaining genetic variability of broodstocks (Tiersch 2000, Lahnsteiner 2004). The technique also ensures preservation of genetic materials of superior wild fish populations and enables gene transfer from wild and hatchery stocks (Cloud et al. 1990, Tiersch et al. 1998).

Conclusions

Genetic stock conservation for wild and domesticated fishes is very important, as the genetic diversity of species develops through non-recurring evolutionary events over millions of years. It is vital in improving the standard of living through sustainable utilization of fish genetic resources in a populous country like Bangladesh. Quality seed production largely depends on the availability of superior genetic material. Therefore cryopreservation techniques can potentially help to preserve and supply quality germplasm from genetically superior broodstocks. Thus, cryogenic techniques can assist in the conservation of biodiversity, to bring back threatened species to natural environment with restocking programs, as well as in improving aquaculture production. Cryogenic sperm banks for fish need to be established as means of germplasm conservation in Bangladesh. The country has a well-established network for the delivery of cattle and goat semen at governmental and non-governmental levels. Through the Department of Livestock (DoL), the government provides frozen cattle semen throughout the country. The largest non-government organization (NGO) of Bangladesh - Building Resources Across Communities (BRAC) presently produces 500,000 doses (straws) per annum and operates through 89 semen stations and 1,279 technicians nationwide to supply cattle and goat semen. If the protocols were properly developed and public and private entrepreneurs can be made aware about the long-term prospects, cryopreserved fish sperm could be integrated with the existing network for country-wide delivery and distribution (Lang et al. 2003).

It is urgent to care for the biodiversity of these beautiful and valuable fishes – the pride, heritage, and livelihood of Bangladesh before they are lost forever. Researchers (in Bangladesh and overseas), policy makers, governmental and NGOs and national and international donors should come forward to conserve the fish species using *in situ* and *ex situ* techniques.

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New Chapter

Status of Fish Germplasm and Cryopreservation in Africa

Kouassi S. Da Costa

Introduction

The genetic resources of African fishes are subject to the risk of permanent loss or reduction of genetic diversity due to cross-contamination of farmed stocks and wild populations because of inappropriate management systems. Degradation of national parks and natural reserves due to the effects of climate change and other human activities (e.g., forest degradation due to urbanization and to agricultural development, alteration of water hydrochemical regimes, misuse of water for irrigation such as the progressive drying of Lake Chad, and various forms of pollution). Overfishing and increasing illegal fishing activities are other constraints for maintaining the genetic diversity of African fishes. Armed conflicts and the destruction of germplasm which ensues such as in Cote d'Ivoire (Ivory Coast) since 2002 (Sangare et al. 2009) are a constant threat. Another indirect cause of fish depletion is the lack of adequate monitoring of fishing activities (Yapi-Gnaore et al. 1998). Of about 2000 African freshwater species, 296 species including 250 cichlids from Lake Victoria are listed as endangered (Thorpe 1995).

These observations lead to inevitable questions about strategies for implementation of conservation programs for sustainable maintenance of fish genetic resources in Africa. This situation is reminiscent of efforts by the international community [FAO Expert Consultation in Rome in 1980, 1981 and 1992 (FAO 1992), and ICLARM-FAO workshop in Rome in 1995 (Pullin and Casal 1996), International Symposium in Vancouver, Canada (Harvey 1998)], which led to the establishment in American, European, and Asian countries of appropriate strategies for *in situ* or *ex situ* fish preservation in the interest of fisheries, aquaculture, and conservation.

For the African continent, questions of conservation of fish genetic resources are an issue because, under the guidance of the African Union summit "Fish for All" (Mbodj 2007), the fisheries sector makes an essential contribution to food security and nutrition for 200 million Africans and provides income for more than 10 million people engaged in production, development, and trade. Fish has also become a major export commodity for Africa. Unfortunately, natural fish stocks are reaching the limits of capture, while aquaculture production remains insufficient to meet burgeoning demands.

Cryopreservation of Fish Sperm in Africa

The International Symposium in Vancouver (Canada) on the erosion of biological diversity of fish and measures for their protection (Harvey 1998) considered, among other things, the principle of taking into account techniques and systems for *ex situ* conservation of fish genes, e.g., cryopreservation, and gene banking in the strategies for germplasm management. So, it is important to consider the use of these techniques for gene conservation of the fishes in Africa.

The main fish species reared on the African continent are: tilapias *Oreochromis niloticus*, *Hemichromis fasciatus*, *Oreochromis mossambicus*, *Tilapia rendallii*, African sharptooth catfish *Claria gariepinus*, mud fish *Clarias anguillaris*, sampa *Heterobranchus longifilis*, African

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catfish *Heterobranchus bidorsalis*, *Heterobranchus isopterus*, common carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mikyss*, black bass *Micropterus salmoides*, silver carp *Hypohthalmicthys molitrix*, grass carp *Ctenopharyngodon idella*, snake-head *Parachanna obscura*, Nile perch *Lates niloticus*, African bonytongue *Heterotis niloticus*, and goldfish *Carassius auratus auratus*. Tiger prawn *Peneaus monodon* is also bred.

Overall, the use of cryopreservation techniques for fish is limited in most African countries. South Africa, based on the number of reported studies (Steyn et al. 1985, Steyn and Van Vuren 1987, Steyn and Van Vuren 1991, Steyn 1993, Van der Walt et al. 1993) is the most advanced African country in this field. These studies addressed freezing, survival, and fertilizing capacity of sperm in the following freshwater fish: Characidae, sharptooth catfish and rainbow trout. Moreover, methods of induced spawning instituted to develop long-term breeding programs for *Pseudobarbus quathlambae* in the Senqunyane, Jordan, and Bokonga rivers (Lesotho) to ensure the survival of this species. For this goal, cryopreservation protocols for sperm of this fish species were evaluated. This program allowed reintroduction of species in an identified sanctuary area (Rall 1989) (Table 1).

Table 1. Sperm cryopreservation studies reported for native species by African countries and institutions.

Country	Institution	Family	Species	Study purpose	Reference
South Africa	Rand Afrikaans University	Clariidae	*Clarias gariepinus* sharptooth catfish	Sperm cryopreservation	Steyn et al. 1985
	Rand Afrikaans University		*C. gariepinus*	Fertilizing capacity of thawed sperm	Steyn & Van Huren 1987
	Rand Afrikaans University	Characidae Alestidae	*Hydrocynus forskahlii* elongate tigerfish *Brycinus imberi* spot tail	Sperm cryopreservation for ornamental use and genetic diversity conservation	Steyn & Van Huren 1991
	Rand Afrikaans University	Clariidae	*C. gariepinus*	Freezing rate effects on survival	Steyn 1993
	Rand Afrikaans University		*C. gariepinus*	Cryopreservation effects on Hardy - Weinberg proportions in progeny	Van der Walt et al. 1993
	Rand Afrikaans University	Cyprinidae	*Pseudobarbus quathlambae* Manuti minnow	Conservation program for *P. quathlambae*	Rall 1999
Cote d'Ivoire	Oceanologic Research Center (CRO / ORC)	Clariidae	*Heterobranchus longifilis* sampa	Cryopreservation research	Oteme et al. 1996
Nigeria	University of Ibadan	Clariidae	*C. gariepinus*	Cryopreservation for fingerling production	Adeyemo et al. 2007

With regard to other African countries, little work has been reported. The few studies available are those of Oteme et al. (1996) in Cote d'Ivoire on the cryopreservation of *H. longifilis* sperm and of Adeyemo et al. (2007) in Nigeria on the evaluation of diluent on sperm motility and sperm viability of *C. gariepinus* (Table 1). This weakness for use of cryopreservation techniques for conservation of African fishes, however, is offset by the international scientific

contribution. One could cite, by way of examples, studies on cryopreservation of Nile tilapia sperm (Chao et al. 1987, Altunok et al. 2004, Musa 2010), sharptooth catfish (Miskolczi et al. 2005), common carp (Cognie et al. 1989), Mozambique tilapia (Harvey 1983), and rainbow trout (Majelac et al. 1990, Maisse 1994, Baulny 1997, Robles et al. 2003).

This indicates that Africa does not need to start from nothing in matters of fish sperm cryopreservation. Data from studies in Africa and elsewhere should be able to provide a foundation for development initiatives of fish cryobanks in African countries.

The Need for Cryopreservation of Fish Species in Africa

Domestic fish production in 50 countries amounted to about 887,567 tonnes in 2008 against about 7,109,824 million tonnes for inland fishery catches (FAO 2008). Of all African countries, only two contribute appreciably to global production of farmed fish (Figure 1). These are Egypt with 11% of world production (Nile tilapia, common carp) and Nigeria with 2% of world production (African sharptooth catfish). South Africa with 1,000 to 1,200 tonnes per year for rainbow trout and 2,000 – 4,000 tonnes per year for African sharptooth catfish (Van der Walt 1993) is far behind. In 2008, South Africa (3,215 tonnes), Madagascar (9,581 tonnes), Zambia (5,640 tonnes), Ghana (5,594 tonnes), Kenya (4,452 tonnes), and Tunisia (3,328) had an appreciable but low aquaculture production (FAO 2008). With regard to other African countries, this production is equal or less than 1,000 tonnes per year.

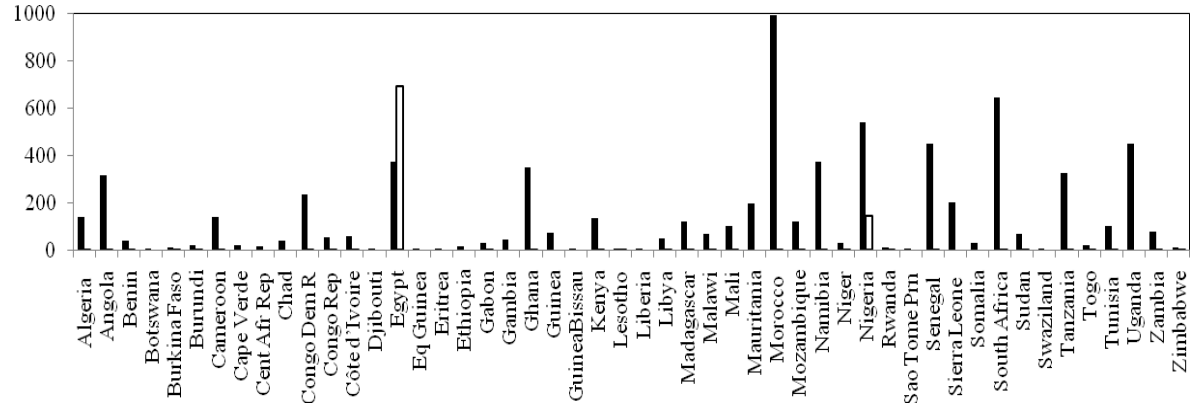

Figure 1. Capture (black bars) and aquaculture (white bars) production in African countries (x 1000 tonnes) (FAO 2008).

African aquaculture production is weak. This may be explained by the fact that the production systems are generally, extensive, semi-intensive and, rarely, intensive. They are characterized by a lack of quality fingerlings for supply to farmers. The lack of availability of this basic input and unreliable quality result in irregular production cycles, fish populations with low capacity for growth due to the reduction or loss of genetic variability (e.g., inbreeding), genetic drift, and reduced production capacity.

This situation calls for decisive change in strategy to ensure effective development of aquaculture in Africa. This requires adoption of systems and techniques for breeding that would ensure production of fingerlings in sufficient quantity and quality for the benefit of farmers and, if necessary, for restocking activities of the major African fisheries and river systems.

For this purpose, as indicated by Altunok et al. (2004), cryopreservation of sperm can be a vital tool in gamete and broodstock management of cultured fish species; and can ensure a stable supply of sperm for optimal use in hatchery production and laboratory experiments. Moreover, rare genetic material can be conserved and transported economically, and materials for research can be made more accessible. Also, the application of electrophoretic data (i.e, biochemical markers) in conjunction with cryopreservation can provide added advantages for conserving genetic diversity in the wild as well as for domesticated fish populations by the reintroduction of rare alleles into affected fish populations (Van der Walt 1993).

In view of the benefits of these techniques, depletion of fisheries resources, needs of restocking and the commitment of African countries (Mbodj 2007) through the New Partnership for Africa Development (NEPAD/Africa Union) action plan, the use of cryopreservation for the conservation African fish has become a necessity. The integration of cryopreservation into production systems is expected to achieve the objectives of the NEPAD plan which involves, among other things, to increase production of fisheries and aquaculture on the continent while maintaining sustainable fishery resources (Mbodj 2007).

Potential for Conservation of Fish Germplasm in Africa

Currently, germplasm conservation of fishes in Africa is mainly based on live populations of broodstock species kept in the National Research Centers, and wild fish fauna preserved in the waters of national parks and natural reserves. If the *in situ* and *ex situ* preservation of fish genetic resources remains a concern, according to the reasons discussed above, signing of the Convention on Biological Diversity (CBD) and the United Nations Food and Agriculture Organization (FAO) Guidelines for Responsible Fisheries (FAO 1998) by most of African countries offers hope of strengthening management activities.

Several advantages are to be considered for the establishment of efficient systems for conservation of fish genetic resources in Africa. These include, notably: *(i)* agricultural policy more and more coordinated through the regional or continental organizations (Table 2; Figure 2); *(ii)* pooling of scientific expertise (FARA, CORAF/WECARD); *(iii)* progressive training of African scientists in USA and European countries in techniques for cryopreservation; *(iv)* reinforcement with FAO assistance of African regional organizations responsible for monitoring of marine water bodies, exploited stocks, and for fighting against illegal fishing; *(v)* anchoring of the national research centers to the networks of International Plant Genetic Resources Institute (IPGRI) for plant genetic resources and to the World Fish Center (WFC /ICLARM) for the fish genetic resources; and *(vi)* existence in the national research centers of *ex situ* conservation devices for plant and animal genetic resources (e.g. *in vitro* seedlings, cryobanks for bovine semen for artificial insemination) that could be drawn upon for the development of fish germplasm in Africa. Knowledge of local peoples should be incorporated into these initiatives.

Perspectives for the Development of Fish Gene Banks in Africa

It is apparent that there is need for better management of fish genetic resources in Africa. To achieve this goal, it is necessary that efforts for resource conservation should be integrated with cryopreservation techniques that have to be supported and encouraged by African regional organizations and the international community. This should now be a priority. Indeed, as indicated by Thorpe et al. (1995), this is needed to avert a loss of many aquatic species. This goal

Table 2. Continental and regional organizations in Africa, that are, or could be, involved in conservation of germplasm and genetic resources.

Organization	Acronym	Website
Africa Union	UA/AU	www.africa-union.org
Common Market of Eastern and Southern Africa	COMESA	www.africa-union.org/root/au/recs/comesa.htm
Community of Sahel – Saharan States	CEN-SAD	www.africa-union.org/root/au/recs/cen_sad.htm
Eastern African Community	EAC	www.africa-union.org/root/au/recs/EAC.htm
Economic community of Central African states	ECAS	www.africa-union.org/root/au/recs/eccas.htm
Economic community of West African states	ECOWAS	www.africa-union.org/root/au/recs/ecowas.htm
Forum for Agricultural Research in Africa	FARA	http://www.fara-africa.org/
Intergovernmental Authority for Development	IGAD	www.africa-union.org/root/au/recs/igad.htm#vision
Southern African Development Community	SADC	www.africa-union.org/root/au/recs/sadc.htm
Southern African Customs Union	SACU	http://www.sacu.int/
Union of Arabic Maghreb	UAM	www.africa-union.org/root/au/recs/uma.htm
West African Economic and Monetary Union	UEMOA	http://www.uemoa.int/index.htm
West and Central African Council for Agricultural Research and Development	CORAF/WECARD	http://www.coraf.org/English/English.html

was also embodied at the 8th Symposium on Fish Reproductive Physiology at St. Malo (IFREMER, France) to save genetic heritages, to maintain fishing activities, to support aquaculture, and to preserve endangered species. (INRA-IFREMER 2007).

In this context, adoption of the NEPAD action plan by heads of states and African governments that met in Abuja (Nigeria) in 2005 for development of fisheries and aquaculture in Africa by improving management of natural fish stocks, increasing investment in critical areas of inland fisheries, coastal marine fisheries, and aquaculture (Mbodj 2007), is an encouraging sign. This should facilitate initiatives for the adoption of techniques of cryopreservation for mass production of juveniles of fish bred in Africa to ensure the availability of animal protein for the majority of African people. Also, the establishment of cryobanks should allow in several African countries, the collection and preservation of sperm from farmed fish and natural water bodies for the purpose of safeguarding and enhancing the genetic heritage of native species.

The question of fish sperm cryopreservation in Africa poses also the problem of genetically modified (GM) crops or fish species. This is a controversial issue. Many African countries do not adhere to the idea of introducing GM in cropping systems and aquaculture. In this matter, Malawi, Zambia and Zimbabwe are exceptions. These countries have introduced legislation to help regulate the GM organisms. Regarding the issue of use of GM organisms, the initiative of the states of the Commons Market for Eastern and Southern Africa (COMESA) which are consulting on development of a biosafety road map to guide national regulations on transgenic organisms, and on regimes and mechanisms for monitoring and inspection (Nature 2010) should illuminate a large number of African countries. This could facilitate adoption of a global approach in Africa for the management of transgenic resources that in one way or another, are already present in culture systems of food crops throughout the continent, and could present a model or organizational basis to assist development of protections for germplasm and genetic resources of aquatic species.

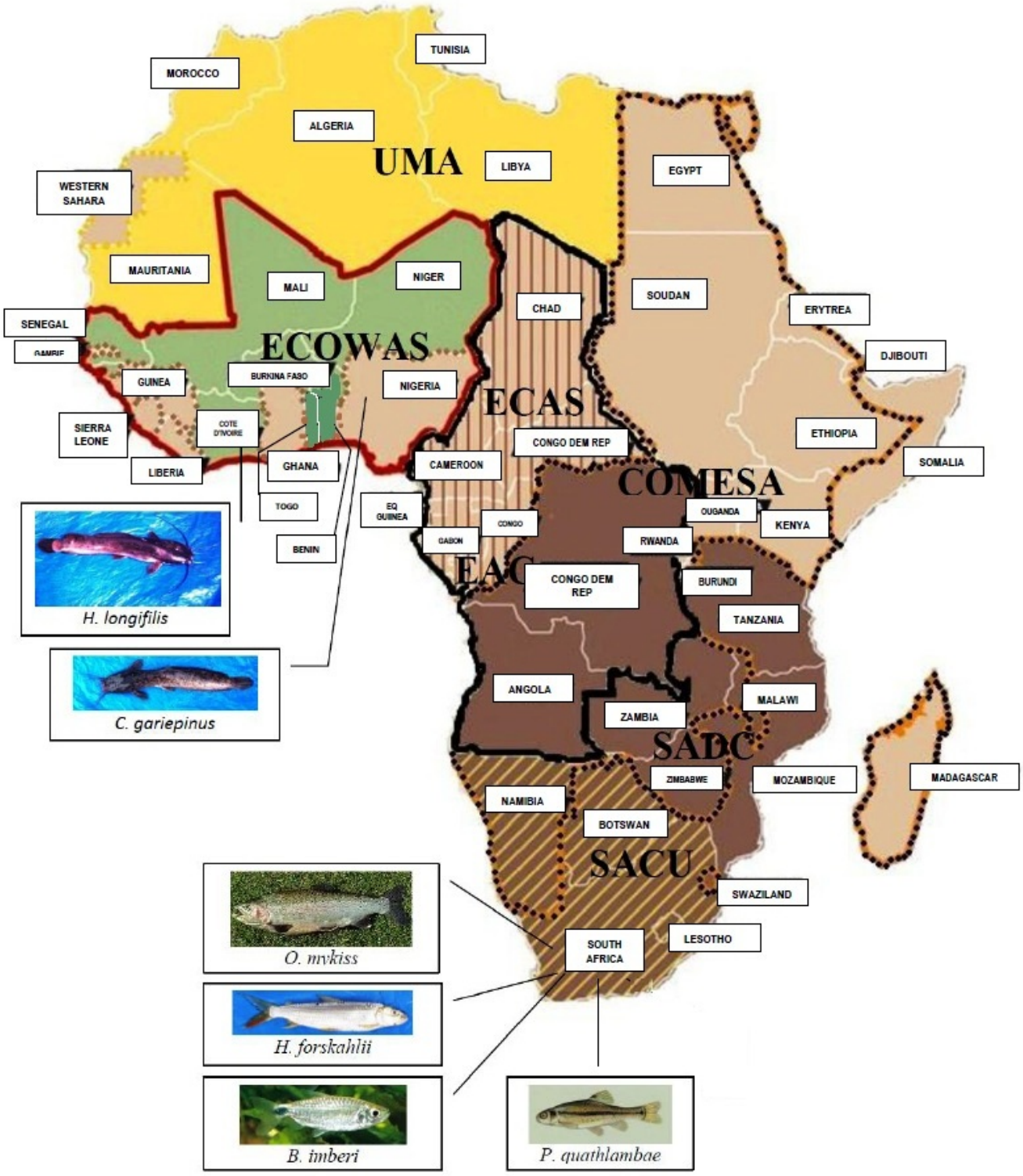

Figure 2. African Regional Organizations, their geographical areas, and countries with published cryopreservation studies in various fish species.

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The Status of Cryopreservation of Aquatic Species in Mexico

Carmen G. Paniagua-Chávez, Gorgonio Ruiz-Campos and Marisela Aguilar-Juárez

The Need for Cryopreservation of Aquatic Species in Mexico

According to the National System of Information on Biodiversity (SNIB), Mexico has the second highest diversity of ecosystems in the world and is fourth in major species richness. Mexico is home to ~500 species important to fisheries, 600 species of plants that are used for reforestation, and some 4,000 species of plants known to contain medicinal properties. Hundreds of exotic species and other thousands with biotechnological potential are included in Mexican ecosystems (SNIB 2007). Despite recognition that Mexico is home to a large and unique biodiversity, the diversity of aquatic ecosystems is not acknowledged as well as terrestrial systems. Mexico is surrounded by four important seas (Pacific Ocean, Gulf of Mexico, the Caribbean, and Gulf of California). These regions contain species richness, endemism and diversity as high as those seen in terrestrial regions (Salazar-Vallejo and González 1993).

Similar to other aquatic ecosystems around the world, the aquatic biodiversity of Mexico is threatened. Tourism development and constant demand for food have placed tremendous pressure on the Mexican coasts and seas (CONABIO-CONANP-TNC-PRONATURA 2007). The principal strategies developed by the Mexican government to conserve aquatic diversity were to assign protected areas and develop protection programs (CONABIO 2006). To date, only 1.4% of the marine environments are safeguarded under the protected area decree (CONABIO 2006). The official records for Mexico indicate that ~1,559 species of animals are registered under the categories of extinct, endangered, threatened or protected (Diario Oficial de la Federación 2007). From these records, 422 species are aquatic species, and 45% are fishes (Table 1).

Table 1. Imperiled aquatic species from Mexico based on official records (Norma Oficial Mexicana 059-Ecol-2001, Diario Oficial de la Federación, 2002).

Group	Extinct	Endangered	Threatened	Protected	Total
Amphibians	0	6	42	149	197
Fishes	11	70	74	30	185
Mollusks	0	9	0	8	17
Crustaceans	0	5	7	3	15
Corals	0	0	0	7	7
Echinoderms	0	0	0	1	1
Total	11	90	123	198	422

Also, the red list of threatened species published by the International Union for Conservation of Nature and Natural Resources (IUCN) lists ~2,945 species of animals from Mexico in one of 9 categories of risk (IUCN 2007). Consequently, the need to conserve aquatic diversity in Mexico is critical. In addition to the strategies currently used by the government, development of

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cryopreservation of gametes and embryos can assist conservation of genetic resources of aquatic species and support conservation programs to safeguard endangered and threatened species.

The paucity of information found on aquatic population dynamics, in combination with the shortage of protected areas and the increasing pressure on aquatic ecosystems due to human activity, make aquatic organisms very vulnerable in Mexico. The status of freshwater fishes has been better documented than that of marine species. One of the native freshwater fishes of northwestern Mexico with a high potential for aquaculture and conservation are the trout species of the genus *Oncorhynchus*. These species are distributed in the headwater streams of the Sierra Madre Occidental (SMO) and Sierra San Pedro Mártir (SSPM), within an altitudinal interval from 560 to 2,640 m above sea level (Ruiz-Campos et al. 2003). This genus is represented in Mexico by at least ten native taxa, of which only two have been taxonomically described: the Mexican golden trout (*Oncorhynchus chrysogaster*) (Figure 1, next page), from the SMO in Chihuahua, and the Nelson's rainbow trout (*Oncorhynchus mykiss nelsoni*), from SSPM in Baja California (Behnke 2002, Ruiz-Campos et al. 2003, Hendrickson et al. 2003, 2006). The conservation status of this complex of species is vulnerable due to their confined distribution and low abundance within populations (Hendrickson et al. 2006, Jelks et al. 2008).

This complex of species represents a spectrum of evolutionary relationship with genetic diversity achieved through 0.5 million years of adaptation and speciation. The genetic pool provided by this myriad of species could be used for genetic improvement and enrichment of the stocks of cultured rainbow trout, and in programs of recovery of endangered or threatened species. One of the main problems that confront commercial culture of exotic rainbow trout (*O. mykiss irideus*) in Mexico and elsewhere in the world is the erosion of genetic diversity of broodstocks due to repeated interbreeding of a limited stock of breeding adults. The effects of the inbreeding are reflected in high mortality due to susceptibility to disease and infections by parasites, and a lessened tolerance to daily changes of temperature and dissolved oxygen.

The genetic diversity and physiological traits of cultured rainbow trout could be gradually improved by the programmed addition of genetic material from the native trout populations originating from the wide geographic range of northwestern Mexico. Therefore, the establishment of a germplasm bank for native trout populations of northwestern Mexico, provides a window of opportunity with several lines of application in the short-term or long-term such as: (1) preservation of genetic pools of different native trout populations, (2) enrichment of genetic diversity of cultured rainbow trout stocks, (3) establishment of a source of genetic material for *ex situ* reproduction of imperiled species, and (4) support of programs focused on reproduction and stocking of trout for sustained sport fishing. Preservation of the genetic resources of these trout species can thus provide a model for many other fish species in Mexico and elsewhere.

The Germplasm Bank of Aquatic Species of Baja California

In 2005, the Germplasm Bank of Aquatic Species (GBAS) was inaugurated in Baja California with the purpose of conserving genetic resources of aquatic species. This project was supported by the Secretary of Environment and Natural Resources (SEMARNAT) and the National Council of Science and Technology (CONACyT). The GBAS began as a pilot project to collect genetic resources from the Baja Peninsula, which is the second longest and most isolated peninsula in the world (Grismer 2000) and contains unique and threatened

Figure 1. (Top) Mexican golden trout (*Oncorhynchus chrysogaster*, 138 mm standard length) from the Sierra Madre Occidental, Chihuahua. (Bottom) Nelson's rainbow trout (*Oncorhynchus mykiss nelsoni*, 148 mm standard length) from the Sierra San Pedro Mártir, Baja California. Photographs by G. Ruiz-Campos.

biodiversity. In the past 5 yr, collections of germplasm from five important biological or commercial species have been performed (Table 2).

Table 2. Cryopreserved material stored in the Germplasm Bank of Aquatic Species of Baja California.

Common name	Scientific name	Status	Material
Totoaba	*Totoaba macdonaldi*	Endangered	Sperm
Rainbow trout	*Oncorhynchus mykiss nelsoni*	Special protection	Sperm
Pacific oyster	*Crassostrea gigas*	Commercial	Oocytes
Red abalone	*Haliotis rufescens*	Commercial	Sperm
California halibut	*Paralichthys californicus*	Commercial	Sperm

Sample collection is subject to reproductive seasonality for each species and government permits are required for collection of samples from endangered or protected species. Totoaba and Nelson's rainbow trout are two endemic species, for which frozen sperm was used to fertilize eggs in 2009. Abalones and flounders are important commercial species that will also benefit from the bank. Frozen sperm from these species will be used for breeding when fresh sperm is not available.

In addition to collections, different experiments need to be performed to determine optimal conditions for freezing of samples. Such experiments are being performed for sperm of white shrimp, *Penaeus vannamei*, and the sea cucumber, *Cucumber apostichopus*, and for sperm and embryos of red abalone *Haliotis rufescens*. The research developed at the GBAS is the product of collaborative work among Mexican universities including the Universidad Autónoma de Baja California (UABC). The Universidad Autónoma Nacional de México (UNAM), The Universidad Autónoma Metropolitana (UAM) and The Centro de Investigaciones en Matemáticas (CIMAT). This research has thus far focused on optimization of freezing protocols, reduction of pathogen transfer, determination of gamete quality, and development of information technologies.

The urgent need to protect the biodiversity of Baja California presents a long list of species that must be considered for cryopreservation. Many mollusks (including all species of endemic abalone, two species of geoduck, and important clams), marine fish (sardines and yellow tail, among others) and freshwater fishes (such as endemic trout) will be cryopreserved.

Perspectives for the Future of Cryopreservation of Aquatic Species in Mexico

In Mexico, previous conservation of genetic resources has been limited almost entirely to commercial plant species. These duties are carried out principally by the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), the Centro Nacional de Mejoramiento del Maíz y Trigo (CIMMYT) and the Centro Internacional de Mejoramiento de Maíz y Trigo of the Universidad Autónoma de Chapingo (UACH). Most of the techniques to conserve these germplasm are based on cold storage of seeds. Cryopreservation programs for livestock are carried out in the Facultad de Veterinaria, Universidad Autónoma de Mexico (UNAM). Most of the cryopreservation is performed for bull, sheep, and horse, however, a well-established germplasm bank for domestic species does not exist although some companies cryopreserve sperm and embryos at small scale for commercial purposes (S. Rincón, Ganadería Romar; E. Tavitaz, Hacienda La Escondida, personal communications).

The large-scale conservation of genetic resources of aquatic species is a new concept and is performed in Mexico only by the GBAS. Recently, efforts have been made by the government, research institutes, universities, and industry, to develop the first National Germplasm Bank (NGB) and to create a National Seed Center, and a Forest Germplasm National Center. The GBAS has been considered to participate in these challenges. This will be a good opportunity to expand the work performed by the GBAS, to cryopreserve important aquatic species from the entire country and to participate actively in the developing conservation programs of Mexico.

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Current Status of Sperm Cryopreservation of Brazilian Characiform Fishes

H. P. Godinho and Ana T. M. Viveiros

Introduction

The order Characiformes includes a large variety of exclusively freshwater fish families and species in Africa and the New World. In the Americas, characiforms are found from Texas in North America throughout Central and South America, encompassing ~1000 species (Buckup et al. 2007) which correspond to about 38% of all known neotropical species. These fishes range in length from a few cm (e.g., glandulocaudines, genus *Mimagoniates*) to over 90 cm (e.g., 'dourados', genus *Salminus*). Characiforms have commercial importance as ornamental species (e.g., small cardinal-tetras, genus *Paracheirodon*), fisheries food resources (e.g., mud-eating prochilodontids, genus *Prochilodus*), aquaculture (e.g., 'pacus', genus *Piaractus*), or sport fishing (e.g., dourados). Today, fingerlings of several characiforms are hatchery produced for aquaculture and stock enhancement (Godinho 2007).

All characiforms that have had sperm cryopreserved to this date (Table 1) are highly fecund, external fertilizers, and migratory, except *Brycon nattereri* for which no information on migratory condition is available. Besides their ecological and practical importance, cryopreservation in characiforms sperm is valuable as it can be used to improve genetic quality of fingerlings produced in Brazilian hatcheries. In addition, some characiforms discussed in this chapter are of special concern because they are under threat of extinction (e.g., 'matrinchãs',

Table 1. Brazilian characiforms studied for sperm cryopreservation during the past decade.

Family*	Species	Common names
Prochilodontidae	*Prochilodus lineatus*	Streaked prochilod, curimba
Anostomidae	*Leporinus elongatus*	Piapara
	Leporinus macrocephalus	Piaussu, piau-açu
	Leporinus obtusidens	Piapara
Characidae	*Brycon amazonicus (=B. siebenthalae)*	Yamú, jatuarana, matrinchã
	*Brycon cephalus (*possibly *B. amazonicus)*	Matrinxã
	*Brycon insignis***	Tiete tetra, piabanha
	*Brycon nattereri***	Pirapitinga
	*Brycon opalinus***	Pirapitinga-do-sul
	*Brycon orbignyanus***	Piracanjuba, piracanjuva
	Brycon orthotaenia (=B. lundii)	Matrinchã
	Colossoma macropomum	Cachama, tambaqui
	Piaractus brachypomus	Pirapitinga, cachama blanca
	Piaractus mesopotamicus	Pacu, pacu-caranha
	Salminus brasiliensis	Dorado

*Following Reis et al. (2003).
**Registered in the national list of endangered species (Brasil 2004).

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'piracanjubas' and 'pirapitingas', all of the genus *Brycon*) caused by dam construction, water quality deterioration, loss of riparian habitats, and overfishing. In this chapter, we review methodologies used to cryopreserve sperm of the Brazilian characiforms published in the last decade and currently in use.

Sample Volume, Concentration, and Motility of Fresh Sperm

Gonadal maturation and spermiation (sperm release) naturally occur in Brazilian migratory fish held in captivity, but hormonal stimulation is a common practice to increase sperm volume. This is usually followed by a decrease in sperm concentration, except for *B. orbignyanus* and *P. mesopotamicus* (Bedore 1999). Most treatments reviewed herein consist of administration of carp pituitary extract, in single or multiple doses. Other hormones, such as human chorionic gonadotropin, have rarely been used in the last 10 years as in *L. macrocephalus* (Ribeiro and Godinho 2003). Hand-stripped sperm volume is highly variable among Brazilian characiforms (Table 2, next page). Some species (e.g., *B. orbignyanus*) yield relatively large volumes of sperm, frequently above 10 mL. Collection of intra-testicular sperm directly from surgically removed testis is routinely performed in *L. macrocephalus*, due to the small volumes obtainable by hand-stripping. The number of spermatozoa per mL is also highly variable, ranging from 4×10^9 sperm per mL in *L. macrocephalus* (Moraes 2004) to 5.5×10^{10} sperm per mL in *P. brachypomus* (Nascimento et al. 2010) after hormone stimulation, and from 1.1×10^{10} sperm/mL in *L. obtusidens* (Taitson et al. 2008) to 4.8×10^{10} spermatozoa per mL in *P. mesopotamicus* (Bedore 1999) without hormone stimulation.

In hatcheries, sperm motility is the main parameter evaluated to assess quality. Preliminary examination of fresh, undiluted sperm is often carried out to identify samples to be discarded because of premature activation due to contamination with water or urine. The most common solutions utilized to induce sperm motility are distilled water, 0.3% NaCl and 1% $NaHCO_3$ (Viveiros and Godinho 2009). The percentage of motile sperm and duration of sperm motility are assessed by light microscopy. Most data for motility duration of sperm are within the typical range for freshwater teleosts, from ~33 sec in *B. orbignyanus* to as long as ~111 sec in *P. lineatus* (Viveiros and Godinho 2009). In *B. siebenthale* (also referred to as *B. amazonicus*), forward movement of the spermatozoa has been recorded by video camera to establish the duration of motility (Cruz-Casallas et al. 2005).

Sperm Cryobanks of Brazilian Fishes

As a consequence of the high fecundity of Brazilian migratory species, the effective breeding numbers used in Brazilian hatcheries for these fishes are quite low leading to a serious reduction of genetic diversity in offspring (Carolsfeld et al. 2003). In this situation, frozen sperm banks would ensure the availability of a large and diverse spectrum of samples thus facilitating an increase in the effective breeding number.

The routine use of sperm cryobanks in hatchery production is still limited. Data on post-thaw viability are highly heterogeneous even for the same species; some reports are incomplete, and given that only positive results are usually published, the true variability of results remains unknown (Viveiros 2005). Thus, development of reliable cryopreservation protocols for fish sperm are often performed on a species-by-species basis. To assist in sharing knowledge,

Table 2. Highest fresh and post-thaw sperm quality of some Brazilian characiforms.

	Fresh sperm[1]			Post-thaw sperm			
Species	Volume (mL)	Concentration ($x10^9$ cells/mL)	Motility (%)	Motility[2] (%)	Fertility (% of control)	Hatching (% of control)	Reference
B. amazonicus	--	--	--	33-39	66-87	--	Velasco-Santamaría et al. 2006
	--	--	80-100	--	--	81-82	Ninhaus-Silveira et al. 2006b
	10.6 ± 0.6	7.6 ± 1.3	92 ± 1	76	31	--	Cruz-Casallas et al. 2006
B. cephalus	4 - >10	9.6 ± 1.6	--	--	--	59-69	Ninhaus-Silveira et al. 2006a
B. insignis	--	24.4 ± 3.8	--	86 [C]	--	--	Shimoda 2004
	3.9 ± 0.7	17.0 ± 7	98 ± 5	86	--	--	Viveiros et al. 2011
B. nattereri	7.6 ± 1.1	30.0 ± 5.6	100	66-72	--	--	Oliveira et al. 2007
B. opalinus	8.0 ± 0.1	41.0 ± 12.9	97 ± 1	81 ± 5	88	75	Orfão 2010
B. orbignyanus	14.5 ± 9.4	10.0 ± 4.3	> 81	20-60	--	--	Bedore 1999
	>10	5.4 ± 2.5	80-100	68	--	77	Maria et al. 2006a
	>10	5.2 ± 2.4	80-100	66	--	--	Maria et al. 2006b
	--	--	80-100	43-45	--	--	Viveiros et al. 2008
B. orthotaenia	2.0 ± 0.8	14.4 ± 2.9	91 ± 10	70 ± 11	--	--	Melo and Godinho 2006
C. macropomum	--	35	80	20-25	88	--	Menezes et al. 2008
	5.0 ± 2.0	22.9 ± 9.8	82 ± 25	56 ± 5 [C]	--	--	Veira 2010
L. elongatus	1-2.5	13.8-15.7	56-77	50-70	67	--	Carvalho 2001
L. macrocephalus	0.4 ± 0.2 [IT; H]	--	--	41 ± 14	84 ± 9	--	Ribeiro and Godinho 2003
L. obtusidens	--	--	80-100	63	--	--	Viveiros et al. 2008
	--	--	80-100	79-86	--	--	Koch et al. 2007
	1.3 ± 0.3	10.9 ± 3.0	93 ± 2	62 ± 14	87	--	Taitson et al. 2008
P. brachypomus	0.83 ± 0.4	3.0 ± 0.6	91 ± 4	78-80	39	39	Fresneda et al. 2004
	13.4 ± 1.5	17.7 ± 1.8	92 ± 1	64 ± 2	2	--	Navarro et al. 2004
	1.8 ± 0.2	55.5 ± 1.4	91 ± 2	81 ± 4 [C]	--	--	Nascimento et al. 2010
P. mesopotamicus	7.2 ± 4.9	36.6 ± 16.5	61-80	20-60	--	--	Bedore 1999
	--	--	--	--	39-86	--	Carolsfeld et al. 2003
	--	--	80-100	79 ± 7	--	--	Orfão et al. 2008
	--	--	75 ± 0.8	16 ± 0.8	--	--	Streit Jr et al. 2006
P. lineatus	1.3 ± 0.8	14.4-20.3	82-90	> 80	-	--	Cruz 2001
	--	--	--	--	80-90	--	Carolsfeld et al. 2003
	--	31.3 ± 10.9	100	69-85	--	7-22	Miliorini 2006
	--	--	80-100	76	--	--	Viveiros et al. 2008
	1.9 ± 0.2	19.2 ± 1.1	> 80	84 ± 4 [C]	77	--	Viveiros et al. 2010
	2.2 ± 0.7	18.1 ± 2.9	100	86-87	95	--	Viveiros et al. 2009b
S. brasiliensis	13.1 ± 3.2	6.2 ± 1.6	80-100	61-64	--	28-38	Viveiros et al. 2009a
	--	--	70-100	79 ± 16	--	--	Nascimento et al. 2008

[1] Carp pituitary-induced spermiation except when otherwise stated, [H] Human chorionic gonadotropin treatment; [2] Subjective motility except when otherwise stated, [C] computer-assisted sperm analysis; [IT] intratesticular sperm; -- data not available; data expressed as mean ± SD where applicable.

emphasis should be placed on standardization of each step in protocols: sperm collection, dilution ratio, extenders, cryoprotectants, equilibration time, cooling rates, thawing rates, and sperm:egg ratios in fertilization tests (Mongkonpunya et al. 2000).

Freezing Media

A freezing medium is an aqueous solution in which sperm are diluted prior to cryopreservation. The freezing medium is composed of an extender and a cryoprotectant agent (CPA). Among the CPA tested in Brazilian characiforms, dimethyl sulfoxide (DMSO) at concentrations of 5-15% has been the most effective (Table 3). However, more recently, methyl glycol has yielded higher post-thaw motility when compared to DMSO in sperm of all species tested (*B. insignis, B. nattereri, B. opalinus, B. orbignyanus, L. obtusidens* and *P. lineatus*) (Table 3, next page), except for *S. brasiliensis* (Viveiros et al. 2009a). In only one study (Navarro et al. 2004) propylene glycol produced higher post-thaw motility compared to DMSO, methanol, glycerol and ethylene glycol. However, when fertility was evaluated, sperm frozen in propylene glycol yielded a rate of only 2% relative to control, while sperm frozen in DMSO or ethylene glycol produced a rate of 27-42%. Egg yolk and powdered milk possess membrane-stabilizing activity; the former is added to the extender typically in combination with glucose when DMSO is used as the CPA. However, due to the difficulty of visualizing sperm cells during motility analysis, several researchers have tested other freezing media without egg yolk and obtained success especially when methyl glycol is used.

Numerous solutions have been used as fish sperm extenders; some are simple saline (0.9% NaCl) or sugar (5% glucose) solutions, while others have more complex formulae, such as BTS™ (Minitub™, Germany), in which salts and glucose are combined. Powdered coconut water (ACP™ 104, developed by researchers at State University of Ceará, Brazil, for freshwater species) has been tested in *B. orbignyanus, L. obtusidens* and *P. lineatus* with success (Viveiros et al. 2008). Coconut water contains salts, proteins, vitamins, carbohydrates, growth factors, phytohormones and saturated fatty acids. The osmolarity of ACP™ 104 is set to 300 mOsmol (Nascimento et al. 2010), while the osmolarity of the other extenders utilized in Brazilian characiforms sperm (Table 3) is 326 mOsmol for 5% glucose (Nascimento et al. 2010), 285 mOsmol for 0.9% NaCl and 318 mOsmol for BTS™ (Maria et al. 2006a). All of these extenders possess an osmolarity within the range of the seminal plasma osmolarity for most freshwater fish species, as osmotic pressures below that induce sperm motility (Alavi and Cosson 2006).

In most of the Brazilian characiforms tested to date, sperm can be successfully frozen in a combination of glucose, egg yolk, and DMSO, at a dilution ratio of sperm to freezing medium of 1:3 to 1.9. Different researchers, however, have defined different freezing media as the best combination of extender and cryoprotectant for the same species (Table 3, next page). The differences in sperm sensitivity to the freezing media for some species may be due to different protocols used to incubate the sperm (such as extender composition, dilution ratio of sperm to freezing medium, or temperature during equilibration). Different methods to assess sperm motility or fertility (such as the ratio of sperm to egg) may also influence the results. Furthermore, it is possible that differences in seminal plasma composition exist between wild and domesticated fish, and among domesticated fish reared under different conditions. These differences may interfere with sperm sensitivity to freezing media (Viveiros and Godinho 2009).

Table 3. Cryoprotectant agents (CPA), extenders, volume of straws, and freezing methods that yielded highest post-thaw sperm quality in Brazilian characiforms.

CPA	Extender	Straw Volume (mL)	Freezing method	Species	Reference
Ethylene glycol	--	0.5	Dry-shipper	*C. macropomum*	Menezes et al. 2008
DMSO	Glucose + egg yolk	0.5	1 cm above LN_2 surface	*B. amazonicus*	Cruz-Casallas et al. 2006; Ninhaus-Silveira et al. 2006b
	Glucose + egg yolk	0.5, 4.0	1 cm above LN_2 surface	*B. cephalus*	Ninhaus-Silveira et al. 2006a
	Glucose + egg yolk	0.5	Directly into LN_2	*P. brachypomus*	Fresneda et al. 2004
	Glucose + egg yolk	0.5, 1.8, 2.5	Dry-shipper	*B. amazonicus*	Velasco-Santamaría et al. 2006
	Glucose + egg yolk	0.5	Dry-shipper	*B. insignis*	Shimoda 2004
	Glucose + egg yolk	0.5	Dry-shipper	*B. orbignyanus*	Bedore 1999
	Glucose + egg yolk	0.5	Dry-shipper	*B. orthotaenia*	Melo and Godinho 2006
	Glucose + egg yolk	0.5	Dry-shipper	*L. elongatus*	Carvalho 2001
	Glucose + egg yolk	0.5	Dry-shipper	*L. macrocephalus*	Ribeiro and Godinho 2003
	Glucose + egg yolk	0.5	Dry-shipper	*L. obtusidens*	Taitson et al. 2008
	Glucose + egg yolk	0.5	Dry-shipper	*P. mesopotamicus*	Bedore 1999, Carolsfeld et al. 2003, Streit Jr et al. 2006
	Glucose + egg yolk	0.5	Dry-shipper	*P. lineatus*	Cruz 2001, Carolsfeld et al. 2003
	Glucose	0.5	Dry-shipper	*S. brasiliensis*	Viveiros et al. 2009a, Nascimento et al. 2008
	BTS	0.5	Dry-shipper	*L. obtusidens*	Koch et al. 2007
	BTS	0.5	Dry-shipper	*P. lineatus*	Miliorini 2006
	BTS	0.5	Dry-shipper	*S. brasiliensis*	Viveiros et al. 2009a
	ACP	0.5	Dry-shipper	*B. orbignyanus*	Viveiros et al. 2008
	ACP	0.5	Dry-shipper	*C. macropomum*	Viveira 2010
Methyl glycol	Glucose	0.5	Dry-shipper	*P. brachypomus*	Nascimento et al. 2010
	Glucose	0.5, 4.0	Dry-shipper	*P. lineatus*	Viveiros et al. 2009b
	Glucose	0.5, 4.0	Dry-shipper	*B. opalinus*	Orfão 2010
	Glucose, BTS, 0.9% NaCl	0.5	Dry-shipper	*L. obtusidens*	Koch et al. 2007
	Glucose, BTS, 0.9% NaCl	0.5	Dry-shipper	*B. insignis*	Viveiros et al. 2011
	BTS	0.5	Dry-shipper	*B. orbignyanus*	Maria et al. 2006a
	BTS	0.5	Dry-shipper	*P. mesopotamicus*	Orfão et al. 2008
	BTS or 0.9% NaCl	0.25, 0.5	Dry-shipper	*B. nattereri*	Oliveira et al. 2007
	0.9% NaCl + egg yolk	0.5	Dry-shipper	*B. orbignyanus*	Maria et al. 2006b
	ACP	0.5	Dry-shipper	*B. orbignyanus*	Viveiros et al. 2008
	ACP	0.5	Dry-shipper	*L. obtusidens*	Viveiros et al. 2008
	ACP	0.5	Dry-shipper	*P. lineatus*	Viveiros et al. 2008, Viveiros et al. 2010
Propylene glycol	Powdered milk	0.5	15 min in N_2 vapor at -76°C	*P. brachypomus*	Navarro et al. 2004

DMSO, dimethyl sulfoxide; BTS™ (Beltsville thawing solution, Minitub): gentamycin sulfate, glucose, sodium citrate, EDTA, $NaHCO_3$, KCl.
ACP™ 104 (Powdered coconut water): contains salts, proteins, vitamins, carbohydrates, growth factors, phytohormones, saturated fatty acids. -- data not available.

Freezing Methods

During the past 10 years, sperm of Brazilian species has been frozen in nitrogen (N_2) vapor, either in straws placed a few cm above the N_2 surface in a polystyrene box, or in portable cryogenic containers known as dry-shippers which can produce a cooling rate of 25-40º C/min to a gradual stabilization at -180º C in 3 min (Taitson et al. 2008). In one study (Fresneda et al. 2004), sperm samples were placed directly into liquid N_2 for vitrification. Sperm has mostly been loaded into 0.5-mL straws, but 0.25-, 1.8-, 2.5- and 4.0-mL straws have also been tested with success (Table 3). In general, cells frozen at fast cooling rates, as in the methods described above, were thawed fast in a water bath at 30 to 60 ºC for 8 to 60 sec (Viveiros and Godinho 2009).

Post-thaw Sperm Evaluation

Post-thaw quality is evaluated mostly as the percentage of motile sperm observed by use of a light microscope. This method of evaluation is subjective, but with practice, technicians can make precise estimations. In most of the species studied, a post-thaw motility range from 20-87% was observed, varying according to the species and freezing protocol used (Table 2). During the last decade, the use of a computer-assisted sperm analysis (CASA) has become popular, but this technology has only recently has been introduced in Brazil. According to the few studies available in the literature, the motility rate evaluated subjectively was similar to that evaluated by CASA in *P. brachypomus* (Nascimento et al. 2010) and in *P. lineatus* (Viveiros et al. 2010). The curvilinear velocity (VCL; ~54 µm/sec) was correlated with fertilization (77% of control) when *P. lineatus* sperm was frozen in ACP™ and methyl glycol (Viveiros et al. 2010). In *B. insignis*, a faster VCL of ~81 µm/sec was observed in sperm cryopreserved in glucose, egg yolk, and DMSO (Shimoda 2004). In *C. macropomum*, a faster VCL of ~35 µm/sec was observed in sperm cryopreserved in ACP™ and methyl glycol (Vieira 2010).

The percentage of live spermatozoa has also been used as a tool to assess post-thaw sperm quality. Sperm cells are stained in eosin-nigrosin (Blom 1950), viewed using a light microscope and can be used to confirm the results obtained with the subjective evaluation of sperm motility, as the results are quite often similar (Maria et al. 2006a, Viveiros et al. 2011). More recently, the use of fluorescent dyes such as propidium iodide and SYBR 14 has become popular as they can be used in combination with flow cytometry.

Post-thaw sperm of Brazilian species have yielded fertilization rates relative to control varying from 31% in *B. amazonicus* to 100% in *P. lineatus* (Table 2). It is important to keep in mind that, when post-thaw sperm is tested for fertility, another source of variation is added: that of egg quality. A control treatment for egg quality, such as fertilizing a batch of eggs with fresh sperm should always be added in a fertilization trial to ensure that low fertilization rates in batches of eggs fertilized with post-thaw sperm are caused by cryopreservation rather than by poor egg quality. Recently, different fertilization rates have been reported in *P. lineatus* eggs fertilized with post-thaw sperm of the same males as high as 83% or as low as 47% depending on the female (Viveiros et al. 2009b). Furthermore, egg quality decreases quickly after spawning (Rizzo et al. 2003). We have observed in *S. brasiliensis* that hatching rate decreases from 80% to 5% in 30 min after spawning when eggs are held at room temperature (24-26º C) (Viveiros and Oliveira unpublished data). The difficulty of controlling egg quality during fertilization trials is

one of the reasons why post-thaw sperm quality is mostly evaluated in term of motility and percentage of live sperm.

Recommendations for Future Work

Considering the high diversity of the Brazilian freshwater fish fauna and the limited data available on sperm biology, which species should be selected for further study? The Brazilian Agricultural Research Agency (EMBRAPA) has recently selected ten native species (the characiforms *B. amazonicus, B. orbignyanus, B. microlepis, Colossoma macropomum, P. mesopotamicus,* and *S. brasiliensis*, the osteoglossiform *Arapaima gigas*, and the siluriforms *Rhamdia quelen, Pseudoplatystoma fasciatu,s* and *Pseudoplatystoma corruscans*), that are potentially important in aquaculture, to receive priority research efforts on reproduction and fry production (Queiroz et al. 2002). Little or no information is available on these topics for *A. gigas, B. microlepis*and *P. fasciatum*. Thus research emphasis should be directed to those species, especially the species for which seedstocks have been commercially produced for aquaculture or restocking.

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XI. Perspectives for the Future Application of Cryopreservation

Seminal Studies in the Cryopreservation of Fish Sperm

John H. S. Blaxter

A major change in the philosophy underlying the activities of biologists took place in the 19th Century. It became clear that there was more to Biology than merely describing the structure and relationships of animals and plants. These organisms should be manipulated to the benefit of Mankind by improved husbandry and especially by selective breeding. Fish were no exception and increasing interest in marine and freshwater hatcheries in the late 19th Century led to a requirement for controlling reproduction, the optimal use of broodstock and the rearing of young life history stages.

Autolysis quickly destroys the internal organs of fish after death. Thus the gonads must be excised or ripe fish "stripped," if some degree of gamete storage is to be achieved. By the 1870's this had been realized for both perch and salmon. Subsequently, a scattering of references in the literature described gamete storage dry, under liquid paraffin and in various diluents, but without any noteworthy result.

Research workers in agriculture were, as usual, ahead of the game. Diluents such as blood serum, sugars, gelatin and buffers were tried (Mann 1954) but Phillip's and Lardy's (1940) discovery of the value of hens' egg yolk plus buffers for extending the viability of mammalian and avian sperm proved to be a minor breakthrough. Fish seemed to present different problems because fertilization was usually external after the eggs and sperm had been released into the surrounding medium.

In the 1940's and 1950's one of the most obvious candidates for fish sperm (and indeed egg) storage were the salmonids. There seemed to be an underlying problem in that sperm remains viable for a very short time once diluted with water. This is not so in the herring (admittedly a less obvious candidate for pisciculture) in which the sperm survives, and can fertilize eggs, after several hr in seawater. Yanagimachi (1953) stored herring gametes in Ringer's solution with resultant viability of 24 hr or more. Would egg yolk as an extender be superior to Ringer's solution? Blaxter (1953) found that egg yolk in borate or phosphate buffers as a diluent allowed fertilization rates in herring of 20% to 30% after 4 d. In fact, this apparent improvement in viability could be matched by storing the gonads dry at 4 °C; after 4 d 50% to 60% fertilization rates could be achieved.

Although this methodology allowed us to bring back herring gametes from distant spawning grounds to the laboratory for rearing experiments, a longer storage time had an additional attraction. It would enable crosses to be made between the numerous physiological races of herring with their widely different spawning seasons, and so help to unravel the complex racial structure of this species.

It was exciting at that time to read about the work of a highly successful group of biologists and biochemists at the National Institute for Medical Research at Mill Hill in London. Among these were J. E. Lovelock (who subsequently went on to develop the Gaia Hypothesis), A. S. Parkes (one of the "fathers" of cryobiology), C. Polge and A. Smith (who later did pioneering work on embryo storage) (Smith 1958). They identified glycerol as a suitable nontoxic antifreeze agent to prevent ice crystal formation in tissues, and quickly appreciated the

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need to stabilize the osmotic pressure of diluent fluids during freezing to and thawing from solid carbon dioxide at -79 °C.

They succeeded first in storing avian sperm (Polge et al. 1949) and then bull sperm for long periods of time (Polge and Rowson 1952). This pioneering work soon led to a new era in animal (and later human) husbandry with the use of artificial insemination techniques. On contacting the Mill Hill team, especially Jim Lovelock, I was told: 1) use as high a concentration of glycerol as possible; 2) use as low an osmotic pressure of the diluent as possible; 3) consider the need to equilibrate the gametes with the diluent, and 4) check freezing and thawing rates exhaustively.

Subsequent experiments (Blaxter 1953) showed that spring-spawning herring sperm frozen in 12.5% glycerol in diluted sea water (1 part distilled water to 4 parts sea water of salinity 3.4%) held at -79 °C was viable after 6 mo when thawed and used to fertilize fresh eggs from an autumn-spawning female. Fertilization rates of 80 to 85% were obtained but no attempt was made to rear the fertilized eggs. Unfertilized eggs did not survive this cryopreservation treatment. Later, Hempel and Blaxter (1961) reared larvae from a cross between Scottish male herring and German female herring where the Scottish herring sperm had been cryopreserved for 6 wk and was used to fertilize untreated fresh German herring eggs.

In the late 1960's, the present extensive work on cryopreservation of fish gametes took off (Horton and Ott 1976). The initial intractability of cryopreserving salmon sperm must have been discouraging in some of the earlier experiments and the important criterion of sperm fertilization ability rather than sperm motility was not used initially. The existence of a whole volume on fish cryobiology shows how pervasive it has become in present-d aquaculture. The value of cryopreservation of gametes and to a lesser extent embryos is clear. The more economical use of sex products, the ability to span space and time, the value in selective breeding and in the storage of genetic diversity make it a powerful tool in our science.

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Annotated Bibliography of Developments in the Last Decade

Much work has been done in past decade to identify factors that influence the quality of fish sperm. These factors include, but are not limited to: temperature, pH, extender composition, and ions. Many tools have been developed to assess the quality of sperm samples including computer-assisted sperm analysis (CASA) and flow cytometry.

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Lessons from the Cryopreservation of Livestock Sperm

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Efforts to freeze spermatozoa began in the mid-1800's with the report of Mantegazza (1866). Modern cryobiology on the other hand began over 100 yr later, with the report that glycerol provided spermatozoa with protection during freezing to -79 °C and through subsequent thawing (Polge et al. 1949). Although the sperm of various mammalian and avian species react quite differently to freezing and thawing, many species have been frozen and thawed on a research scale, but few have been frozen for commercial practice. Some of the greatest advances in genetic improvement in the livestock industry have been made with cattle, where modern cryobiology came together with artificial insemination (AI). Artificial insemination in livestock was fairly well advanced in 1949, making use of stored liquid semen for delivery to farms and allowing rapid application of sperm cryopreservation. Consequently, the dairy cattle industry enjoys the widest possible application of cryopreservation with freezing of bull spermatozoa which is considered state-of-the-art for all species. It is particularly useful in cattle due to the benefits with respect to a long generation interval. Currently 500 to 700 straws of semen can be prepared from a single bull ejaculate compared to 300 to 500 just 15 yr ago. This considerable improvement in efficiency is due to improved sperm harvest methods, and a better understanding of processing, freezing and thawing procedures. Compatibility of frozen semen with management of the dairy herd is advantageous, as is the fact that selection of bulls on the basis of milk production of their daughters is an advantage that is not necessarily repeatable with other species. No other species seems to have so many things going for it with respect to propagation.

Success in the cryopreservation of sperm can be best judged in cattle. The general view held in the 1950's, as frozen bull semen came into commercial use, was that success in other domestic species (e.g. swine or sheep) must follow the progress made over the yr in freezing bull semen by the AI industry and seek to emulate their success. While it was a worthy goal to work toward such an achievement, it was not the kind of approach that brought success to other species. The following comparison with respect to the pig illustrates the effect of such "wisdom" on the success achieved with swine semen preservation. The differences among species in the response of their sperm to various nuances of the freezing and thawing process turned out to be critical. After the success shown with freezing of bull semen, and the subsequent success achieved in bringing it into commercial practice, there was considerable effort put forth to use the same recipes for the pig. The approach was not successful and actually there was considerable frustration since initial post-thaw sperm survival based on sperm motility seemed promising enough to suggest fertilization could be achieved. However, fertilization was not successful. This led to a greater effort over several yr to diverge from bull sperm protocols to focus on developing a strategy specifically for the pig. It is safe to say that the buffer systems needed for mammalian sperm are generally close from one species to another. However, the membrane structure and the ejaculation environment of the semen can be drastically different (e.g. in the boar, ejaculate volume is often greater than 250 mL compared with 5 to 8 mL for bull ejaculates).

One of the more critical factors in the development of cryopreservation of pig sperm was the recognition that acrosomal membranes were being damaged by freezing and thawing. This

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led to classification systems for determining the extent of the damage. Pursel et al. (1972) described such a classification system that was in essence a morphological evaluation of a fixed wet smear. Previous to this, numerous investigators in many species had used the eosin nigrosin live/dead stain to evaluate the quality of ejaculates. However, that method involves a dried smear, which leads to significant artifacts and thus a loss of accuracy. In our laboratory, the acrosome morphology assessment became and remains the standard to evaluate swine sperm, and to monitor sperm damage during processing. This evaluation ultimately led to the first litters produced from frozen boar semen in 1971 after intra-cervical insemination (Pursel and Johnson 1971) followed by the development of a commercial procedure, the Beltsville Pellet Freezing and Thawing Method (Pursel and Johnson 1975). Also, Westendorf et al. (1975) published a commercially adaptable method using a 5-mL maxi-straw. Both methods are still used commercially. Simultaneous to the 1971 breakthrough was similar research by other groups (Crabo and Einarsson 1971, Graham et al. 1971). These groups also directed their attention to the peculiarities of the pig rather than following the protocols for bull sperm. Another factor reported around that time was that glycerol level in the pig was critical. The initial work with cattle had used high glycerol levels, in the 7 to 15% range. Polge (1985) found that high glycerol in the uterus of the pig was detrimental, but that if sperm were put directly into the oviduct, the effect was not a problem with respect to fertility, and fertilization was obtained from oviductal insemination of such semen. Based on this information, the 1971 successes utilized only 2% glycerol and currently the recommended level for swine semen is 3 to 4%, but not higher.

Where are we today with pig semen? Is it optimum? The answer is no. Fertility with frozen swine semen is not yet optimal, even though it is used for export and for market hog production where liquid semen is difficult to transport (Almlid and Hofmo 1996). Research continues into producing frozen semen applicable to everyday swine reproduction. Thus, I think that it may be safe to conclude that the success with bull semen freezing and the tendency to follow that recipe actually delayed the ultimate success in deep freezing boar semen.

The lesson that can be learned from what happened with respect to swine and cattle is clearly one that can be applied to fish cryopreservation; that is, the basic strategy must be defined for the particular species in question. Research approaches can easily become bogged down in searching out details for a tangential hypothesis, or worse yet, modifying methods used for another species without a hypothesis.

The quality of preserved semen is of utmost importance if one is to have a chance at successful fertilization. Sperm motility, as mentioned above, is useful, but does not tell the whole story. Acrosome morphology evaluation was helpful for the pig, but not critical for the bull. There are some species where it could likely not be used at all, because visualization of the acrosome is not easily accomplished (e.g. chicken and turkeys). The functionality of the sperm under *in vitro* conditions has been the subject of considerable research effort and interest, particularly as single cell methods of assessing sperm function have become available (e.g. flow cytometry). Combining flow cytometry with various fluorescent stains for certain aspects relating to sperm function has been helpful in gaining a greater understanding of how sperm function and how the sperm react to changes in their environment. This has led to a greater measure of quality assessment than was possible with the standard microscopic and morphologic characteristics.

Some of the more useful functional techniques that have been applied to mammalian sperm are likely to also be applied to fish sperm. For example, determining live and dead cells on the basis of DNA staining; relating that to membrane integrity, membrane potential, calcium

influx and efflux, chromatin structure and sperm motility have been useful. Several fairly recent reports illustrate the progress in livestock with the use of various fluorescent stains and flow cytometry to test sperm function (Almlid and Johnson 1988, Garner et al. 1994, Evenson and Jost 1994). Assessing sperm motility has always been variable. However, variability in estimation has been reduced in several species with the use of computer-assisted analysis. The computer-assisted analysis system applied by several manufacturers is becoming increasingly reliable as a quantitative measure of sperm movement in a large sample size. However, even with all of these advances, no single *in vitro* test has yet proven to be the 'holy grail' for assessing sperm quality. Using the combination of several *in vitro* measurements will give a good assessment of sperm viability before and after cryopreservation. It would appear that the various *in vitro* methods currently available and those to be developed along similar lines represent the most useful avenue of viability assessment for thawed fish sperm. Several of these evaluation techniques have been adapted to fish cryopreservation in one form or another already.

Should there be concern about cryopreserving sperm and the potential detrimental effects on the offspring produced? Evidence to date suggests that there is no effect on such offspring. Here the cattle industry is the best illustration. Offspring have been born from frozen bull sperm for nearly 50 yr entailing millions and millions of calves. To my knowledge there has never been a report on the detrimental effects of cryopreservation. No other species can claim the magnitude of data available from cattle. However, there has been no indication of problems associated with cryopreservation in other species including humans. It would seem that in our present world, safety through cryopreservation is the widespread assumption in terms of perception and in terms of reality. At the present time, that is also the case for those species where embryo preservation is successful. Genetic quality of offspring from cryopreservation of sperm or embryos has never been the subject of major concern by the animal industry. We are very fortunate in that regard given the various concerns expressed about using modern biotechniques to produce the world's food supply.

What is the impact of embryo cryopreservation in the animal industry? Again, efficiency and specialization and the benefits of the technology are the key factors. Being able to transfer maternal genetics is an advantage particularly for export. Again, how far it is developed is somewhat dependent on the species. Embryo transfer using cryopreserved embryos is done worldwide in the cattle industry. It cannot be done yet in the swine industry, since there are still problems in producing viable embryos from cryopreservation or vitrification. All this is to say, that if the technology is effective and useable, it will ultimately be used. But it must be easily applied. In the cattle industry, cryopreservation success came before non-surgical transfer. The latter was the key to making it widely used. Once embryo cryopreservation becomes available to the swine industry, there will be a need for the least costly methods of delivery and non-surgical transfer. Without them, embryo cryopreservation will not become a major factor in swine production.

How has the animal sperm industry changed in the past 50 yr? As much as one could expect when considering the progress made in mechanics, computers, software, instrumentation, etc. The animal sperm industry has indeed taken advantage of new ideas from other industries such as ampules to straws, cryovials, semen processing machines that use computer chips and increased speed of processing. The improved efficiency that is still occurring has been the result. The effects of the high cost of labor has driven the industry into consolidation and cost cutting much like any other industry in the 20th century. As usage and need grows, so must the effort to develop improvements that will make technologies more economical. These same factors will

drive cryopreservation efforts in fish. Another example from the swine industry might be helpful to make this point. The basic tools for AI of swine were developed in the late 1940's and early 1950's. Subsequently, appropriate diluters for liquid semen storage and shipment were developed in the 1960's and 1970's. With these developments, AI progressed significantly in Europe, but not in North America where adaptable AI technology and AI semen storage technology have been available since the late 1960s. Only in the last 5 yr, however, (about 25 to 30 yr after effective technology was available) did the swine industry avail itself of it in North America. Why did it take so long? Several reasons can be cited. The older producers have left the industry and the younger producers now involved are more receptive to implementing new technology. Economics has also been a factor. It is less costly to use AI than to keep costly boars on the farm. Meat packers place greater emphasis on carcass yield which demands better quality genetics that are now more readily available from the AI boar, and the move towards contract farming with larger operations demands greater efficiency. Could the same sequence occur in the fish industry? Surely it could. But in my estimation, the fish industry is already based on size, efficiency and various types of contract farming. That is to say, the fish industry in the 21st century should be poised to adopt new technology much more readily than the swine industry of the 20th century.

In closing, I would like to say that as research interests wax and wane, there is a need for continuity, and that requires scientists to keep the research areas in the forefront and for scientists and technical industry alike to remain interested in learning new developments. We saw a lack of continuity in the semen preservation area with respect to swine. Our answer was to organize international conferences to achieve a common framework to make advances and to stimulate thinking and new research. It was an effort to bring the world industry and the world science together for discussion and gathering of intimate knowledge of each other's concerns and requirements. I have referenced the three volumes that are available from the three international conferences that resulted from our original idea, with the hope that they might be useful for broadening the research knowledge of investigators in other species (Johnson and Larsson 1985, Johnson and Rath 1991, Rath et al. 1996). The preparation of this volume is evidence to me that scientists involved in fish sperm cryopreservation recognize that need.

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Annotated Bibliography of Developments in the Last Decade

Cryopreservation of livestock sperm is well commercialized. A number of the advances in fish cryopreservation have resulted from advances first discovered and developed within livestock species. The commercialization of aquatic species sperm cryopreservation lags behind the livestock industry. New assessments in technology and economic investigations in marketing have allowed for new lessons to be learned and applied within the field of cryopreservation in aquatic species. Further advances within research, government, and private industry would be well served through further collaboration and open communication.

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Economics and Marketing of Cryopreserved Fish Sperm

Rex H. Caffey and Terrence R. Tiersch

"Frosty," the first calf born from the application of frozen sperm, is often credited for initiating the use of cryopreserved sperm in artificial insemination (AI) programs for dairy cattle. Although research into sperm cryopreservation had been documented almost 50 yr prior, Frosty represented an application of the technology with immediate commercial potential (Herman 1981). Since that time, frozen bull semen has been adopted by AI programs worldwide due to in part to economic benefits such as increased availability of semen and reduced transportation and holding costs.

We can consider the developmental chronology of mature markets for dairy bull sperm as a theoretical trajectory of the market life cycle for cryopreserved fish sperm (Figure 1). The current position on this trajectory indicates a market for fish sperm somewhere beyond conception (based on 40 yr of prior research) and before infancy (early stages of commercialization). What impetus will be required for commercial expansion of cryopreservation with the gametes and embryos of aquatic species? Is it realistic to rely on the emergence of an aquatic equivalent of Frosty? Indeed, fish have been produced experimentally with cryopreserved sperm for over 40 yr, yet no viable markets for frozen sperm currently exist for applications in fisheries and aquaculture.

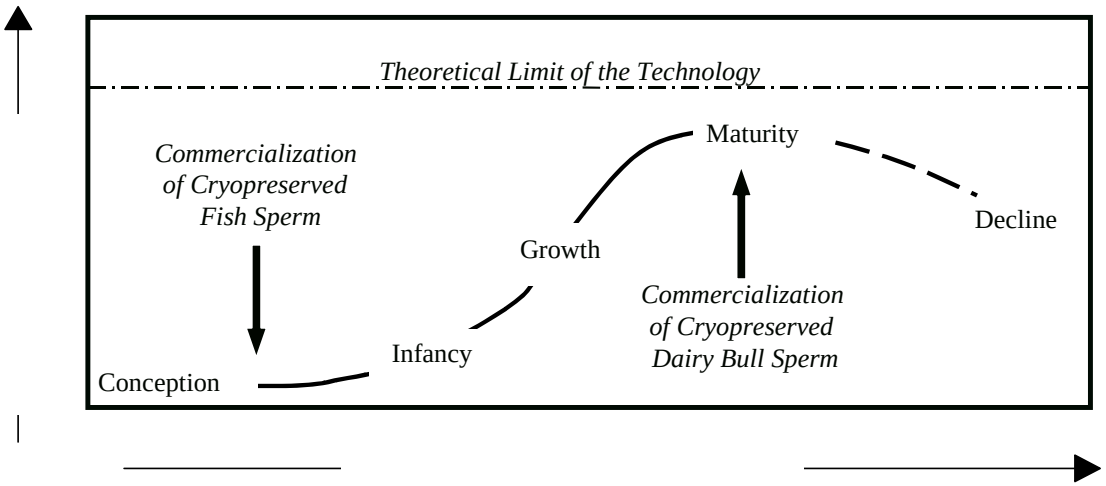

Figure 1. Conceptualized market life cycle for application of technology. Cryopreservation of dairy bull sperm is an example of a mature technology, while the commercialization of fish sperm cryopreservation is currently somewhere between conception and infancy (adapted from Roussel et al. 1991).

In this chapter we discuss some primary economic constraints to the commercialization of sperm cryopreservation for use with aquatic species. Specifically, we review a recent economic study that outlines the basic costs required to integrate sperm cryopreservation into existing fish hatcheries (Caffey and Tiersch 1999). Until recently, such basic information had never been documented, although it is crucial for a realistic evaluation of the feasibility of cryopreservation with the gametes and embryos of aquatic species. Based on this economic data

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and drawing again from the dairy bull model, we provide some preliminary marketing criteria that may be useful for identifying aquatic species where commercialization of cryopreserved sperm is most likely. These criteria provide the framework for a brief case study intended to illustrate how commercialization of research protocols for one application might provide impetus for future markets in cryopreserved fish sperm. Finally, we conclude with a discussion of some conceivable scenarios of the structure and development of future markets for cryopreserved fish sperm.

Economic Considerations: How Much Does it Cost?

A question frequently asked of the cryopreservation practitioner is *"How much does it cost?"* This simple query is becoming increasingly valid as the technology moves out of research and into application with aquatic organisms. However, the question is often difficult to answer, possibly because of variation in protocols, among and within species. Despite the lack of standardization, most methods share similar equipment and common procedures. A representative model of the generic characteristics of sperm cryopreservation with aquatic species may be useful for addressing fundamental economic considerations.

A Generic Model

In a recent study, we developed partial budgets for the integration of sperm cryopreservation into existing fish hatcheries (Caffey and Tiersch 1999). This involved the delineation of generic activities common to programs of fish sperm cryopreservation (Figure 2).

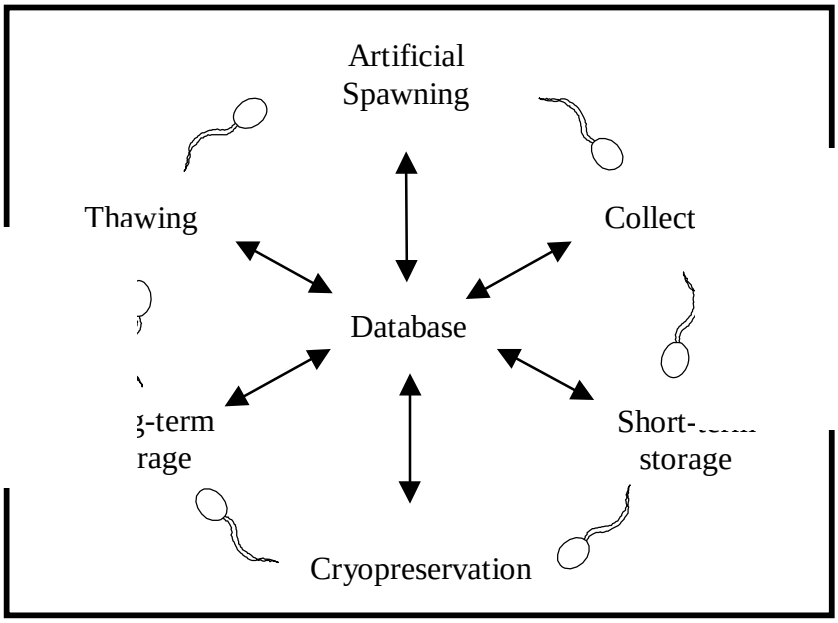

Figure 2. Generic activities of fish sperm cryopreservation. Consecutive components are delineated by a clockwise flow of sperm and two-way arrows are used to indicate maintenance of a centralized database for information on motility, fertilization, and inventory.

Specific activities include: 1) on-site and field collection of sperm; 2) short-term storage (refrigeration); 3) cryopreservation; 4) long-term storage; 5) thawing, and 6) artificial spawning.

Two-way arrows represent information collected during each activity and stored in a centralized database.

Integration Scenarios

To employ the generic model for cost estimation, we must first identify the equipment and supplies utilized at each stage of the cryopreservation process. However, some of these items are not exclusive to cryopreservation and may be found at a given hatchery depending on its range of effort. Additionally, methods for fish sperm cryopreservation range from inexpensive and simple to costly and complex. Therefore, the costs of investing in cryopreservation ultimately depend on the current scope of a hatchery (i.e. equipment on hand) and the level of sophistication desired for a cryopreservation program (e.g. from pure production to pure research). Costs within this range are further defined by whether the hatchery is privately owned or publicly operated.

Few private fish hatcheries currently utilize sperm cryopreservation; yet commercial application should increase, as protocols are refined. However, public hatcheries are often financed by state or federal budgets and have the resources to invest in projects with undocumented technical and economic feasibility. Public hatcheries considering this technology may include a broader range of effort, ranging from small state-run hatcheries servicing put-and-take fisheries, to large-scale research hatcheries such as the Regional Fish Technology Centers operated by the U.S. Fish and Wildlife Service.

Budgeting Assumptions

The costs of equipment and supplies for fish sperm cryopreservation can be estimated using a modified partial budgeting procedure. Partial budgeting is traditionally used to estimate the commercial effects on costs and returns resulting from changes in management, investment, or technology (Shang 1990). Because fish sperm cryopreservation is currently non-commercial, budgeting information is limited to the cost data generated at public institutions. Our study abbreviated the traditional partial budget into a cost-analysis of the investment and operating expenditures necessary for implementing sperm cryopreservation at public and private fish hatcheries.

Physical Capacity

Developing the generic budget required assumptions. For example, the species-independent analysis precluded estimation of fertilization units. Instead, "production units" were defined in the budget as single, 0.5-mL straws of frozen sperm. Straws of this size are commonly used in fish sperm cryopreservation and with the sperm of other animals such as cattle. Setting a standard size for production units allowed extrapolation of those costs directly related to storage capacity. Capital and operational costs were generated for each component of the generic activity model (Figure 2) and expressed for public and private hatcheries at three levels of production (3,000, 6,000 and 9,000 units) which represented the purchase of three levels of storage capacity (1, 2 or 3 35-L storage dewars).

Financial Assumptions

Miscellaneous costs were defined in investment and operating budgets as 5% of budgeted items. Equipment depreciation was charged as facility maintenance and calculated using a straight-line method at 10% per yr with no salvage value. For this analysis, private hatcheries

were assumed to finance their initial investment with an 5-yr intermediate loan at a 10% annual percentage rate (APR) and a charge of 12% APR for operating capital. Private hatcheries were also assumed to pay an 8% local sales tax on all purchases. All prices (reported in $US) represent the mean of three commercial estimates collected from equipment and supply vendors in 1999 (i.e. Parsons Air-Gas, Southland Cryogenics, Tech Air, Sigma, Scientific Products, Curtis Matheson Scientific and VWR Scientific).

Budgeting Results

The following synopsis provides summary information only. For additional information readers are encouraged to review Caffey and Tiersch (1999).

Investment Costs

Investment costs were classified as required or optional to the cryopreservation process (Table 1). Required equipment included items used exclusively for cryopreservation, storage and transport of fish sperm. Total investment for the required equipment ranged from $5,460 to $10,458 for public hatcheries and from $9,497 to $18,190 for private hatcheries.

Optional equipment included items that enhance quality control in the production of cryopreserved fish sperm. The most expensive item was the controlled-rate freezer. This device allows precise control of freezing rates and offers the greatest benefit to research, but would be useful where quality control is required. Total investment for required and optional equipment ranged from $37,290 to $42,288 (public), and $63,039 to $71,488 (private) for production capacities of 3,000 to 9,000 straws.

Operating Costs

Annual operating costs were estimated for production capacities of 3,000, 6,000 or 9,000 straws per yr (Table 2). Chemical expenditures represented a significant portion of the operating budget. For extenders, reagent-grade chemicals were included to provide ingredients sufficient to mix 50 L of Hanks' balanced salt solution (HBSS). Variations of HBSS have been used successfully with the sperm of multiple fish species (e.g. Tiersch et al. 1997) and these same ingredients can be used to formulate many other extenders used for fish sperm. Four commonly used cryoprotectants were budgeted: 1) dimethyl sulfoxide (DMSO); 2) dimethyl acetamide (DMA); 3) methanol, and 4) glycerol. Extracellular cryoprotectants such as egg yolk and milk are also commonly used, but their costs were negligible. Labor represented the largest single component of operating costs in the analysis. We assumed the use of a part-time technician at $10 per hr for manual filling and freezing of straws. However, labor costs would decline if automated straw fillers and freezers can be adopted from their use in the cryopreservation of bovine sperm. Annual operating costs ranged from $4,768 to $10,608 (public), and $5,768 to $12,831 (private) for straw capacities of 3,000 to 9,000 production units.

Table 1. Capital costs for integrating cryopreservation into existing fish hatcheries.

Item		Unit price	Storage capacity (0.5-mL straws) 3,000	6,000	9,000
Required equipment					
Storage dewar (35-L, high capacity)		$945	$1,890	$2,835	$3,780
Roller base for storage dewars		160	320	480	640
Low-level alarms (storage dewars, 115 v)		435	870	1,305	1,740
Shipping dewar (4.3-L, spill proof)		565	1,130	1,695	2,260
Cases for shipping dewars		275	550	825	1,100
Transfer hose and phase separator		190	190	190	190
Thermometer (digital, hand-held, +/- 100^{o}C)		250	250	250	250
Subtotals			$5,200	$7,580	$9,960
Miscellaneous (5%)			260	379	498
Sales tax (8%)			437	637	837
Interest on capital (10%)			3,600	5,248	6,895
Total investment (required equipment only)	Public		$5,460	$7,959	$10,458
	Private		$9,497	$13,843	$18,190

Table 1 Continued. Capital costs for integrating cryopreservation into existing fish hatcheries.

Item	Unit price	Storage capacity (0.5-mL straws) 3,000	6,000	9,000
Optional equipment				
Pipetor (1-10 μL)	227	227	227	227
Pipetor (10-100 μL)	227	227	227	227
Pipetor (100-1000 μL)	227	1,212	1,212	1,212
Water Bath (8-16 L, temperature to 90°C)	1,212	1,249	1,249	1,249
Analytical balance (0.01 g readability, 1500 g max)	1,249	1,350	1,350	1,350
Data logger (hand-held, 5 inputs)	1,350	1,460	1,460	1,460
Distilled water source (2L per hr)	1,460	4,681	4,681	4,681
Vapor pressure osmometer (0-200 mOsmol/Kg)	4,681	7,181	7,181	7,181
Laboratory microscope (dark-field, 200-X)	7,181	12,500	12,500	12,500
Controlled-rate freezer	12,500			
Subtotals		$35,514	$37,894	$40,274
Miscellaneous (5%)		1,776	1,895	2,014
Sales tax (8%)		2,983	3,183	3,383
Interest on capital (10%)		22,766	24,291	25,817
Total investment (required and optional equipment)	Public	$37,290	$39,789	$42,288
	Private	$63,039	$67,263	$71,488

Table 2. Annual operating costs for integrating cryopreservation into existing fish hatcheries.

Item	Unit price	Storage capacity (0.5-mL straws)		
		3,000	6,000	9,000
Straws (0.5-mL)	0.06	180.00	360.00	540.00
Goblets	0.26	156.00	312.00	468.00
Canes	0.21	16.38	32.76	49.14
Sealing powder (PVC, 1 Kg)	42.00	13.99	28.14	42.00
Ingredients for HBSS (ACS grade, 500 g of each)				
NaCl	23.38	7.72	15.66	23.38
KCl	23.13	7.63	15.50	23.13
$CaCl_2 \cdot 2H_2O$	48.50	16.01	32.50	48.50
$MgSO_4 \cdot 7H_2O$	45.24	14.93	30.31	45.24
Na_2HPO_4	38.65	12.75	25.90	38.65
KH_2PO_4	35.81	11.82	23.99	35.81
$NaHCO_3$	16.32	5.39	10.93	16.32
$C_6H_{12}O_6$	21.74	7.17	14.57	21.74
Cryoprotectants (500 mL of each)				
Dimethyl sulfoxide (DMSO)	50.00	16.50	33.50	50.00
Dimethyl acetamide (DMA)	50.00	16.50	33.50	50.00
Methanol	50.00	16.50	33.50	50.00
Glycerol	50.00	16.50	33.00	50.00
Liquid nitrogen	116.00	459.36	932.64	1392.00

Table 2. Continued. Annual operating costs for integrating cryopreservation into existing fish hatcheries.

Item	Unit Price	Storage capacity (0.5-mL straws) 3,000	6,000	9,000
Tank rental	37.33	37.33	37.33	37.33
Cryovials (1.2-mL, case of 500)	185.00	61.05	123.95	185.00
Centrifuge tubes (15-mL, case of 500)	148.33	48.95	99.38	148.33
Centrifuge tubes (50-mL, case of 500)	195.67	64.57	131.10	195.67
Microcentrifuge tubes (1.5-mL, pack of 1000)	40.37	13.32	27.05	40.37
Pipettor tips (small, pack of 1000)	47.07	15.53	31.54	47.07
Pipettor tips (large, pack of 1000)	56.00	18.48	37.52	56.00
Sterile filters (0.22-μm; case of 12)	56.80	18.74	38.06	56.80
Disposable sterile bottles (500-mL, case of 100)	56.20	18.55	37.65	56.20
Type-T thermocouples	31.67	63.34	126.68	190.02
Cryo gloves	100.67	201.34	201.34	201.34
Safety goggles	7.67	15.34	15.34	15.34
Labor				
Technician (per hr)	10.00	2400.00	3600.00	4800.00
Facility maintenance	0.10	589.68	859.57	1129.46
Subtotals		$4541.37	$7334.90	$10102.84
Contingency (5%)		227.07	366.75	505.14
Sales tax (8%)	0.05	381.47	616.13	848.64
Interest on operating capital (12%)	0.08	617.99	998.13	1374.80
Annual operating costs	0.12 Public	$4,768.43	$7,701.65	$10,607.99
	Private	$5,767.90	$9,315.91	$12,831.42

Per Unit Costs

In general, as production capacity increases, per unit costs decrease until production is maximized for a given level of technology. A per unit analysis can be used to identify economies of scale for various production capacities. For example, Tisdell et al. (1993) used per unit analyses to identify scale economies for seed production of the giant clam *Tridacna gigas*.

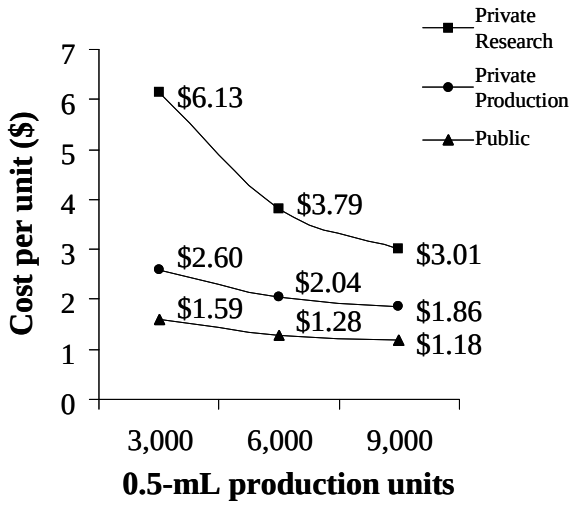

Figure 3. Costs per production unit for cryopreserved fish sperm.

Per unit costs for maximum production capacities of 3,000, 6,000 and 9,000 straws were expressed for three scenarios: 1) total annual costs of private hatcheries investing in required and optional equipment (identified as Private Research); 2) total annual costs of private hatcheries investing in required equipment (Private Production), and 3) operating costs at public hatcheries (Public) (Figure 3). In each case, negatively sloped cost curves indicate economies of scale at increased production levels. Private Research hatcheries exhibited the greatest per unit cost reductions for expanded production capacities. The per unit costs for these hatcheries was $6.13 per straw at 3,000 units, but as production capacity expanded to 9,000 units, per unit costs fell to $3.01 per straw. By comparison, Private Production hatcheries had substantially lower per unit costs because of their lower initial capital investment. These facilities had costs ranging from $2.60 per straw (3,000 units) to $1.86 per straw (9,000 units). Finally, per unit costs for Public hatcheries were calculated using operating expenditures only. Public hatcheries do not typically borrow funds to finance such projects and thus annual costs contain no amortization of initial investments. Accordingly, per unit costs for Public hatcheries were lowest of all, ranging between $1.59 per straw (3,000 units) to $1.18 per straw (9,000 units).

Summary of Generic Cost Analysis

Commercial hatcheries implementing a cryopreservation program can expect to spend as much as 70% more on initial investment and 20% more on annual operating costs compared to public hatcheries. This higher cost structure is due to sales tax and interest, which can account for over 50% of the final investment of private hatcheries. As production expands beyond a given storage capacity, private hatcheries can expect associated increases in required equipment expenditures such as for storage. However, purchases of optional equipment only serve to

improve quality control potential and such equipment can increase initial costs by as much as 300%, while yielding no additional output.

Within the boundaries of pure production and pure research, individual fish hatcheries implementing a cryopreservation program would incur costs specific to the scope of their facility. For example, small-scale commercial fish hatcheries are often production-oriented, placing no effort in research. Cryopreservation programs established at these hatcheries would probably utilize required equipment only. Conversely, large state or federal hatcheries interested in developing a research-oriented cryopreservation program might have much of the required and optional equipment on hand, and thus a research program could be established for a lower investment.

Economies of scale were identified for increased production in three scenarios. Expanding the production of cryopreserved fish sperm at these hatcheries from 3,000 to 9,000 units resulted in per unit cost reductions of 25% to 70% (private research), 27% (private production) and 23% (public hatcheries). However, it is important to note that per unit costs in this analysis represent the minimum cost per straw possible for three levels of maximum storage and production capacity. An individual hatchery's cost structure and annual production of cryopreserved sperm will ultimately determine the actual per unit costs.

To summarize, it is logical that public fish hatcheries will be more likely to invest in cryopreservation in the near future because of lower investment costs and their insulation from the economic risks associated with new technology. However, increased commercialization of cryopreserved fish sperm can be expected as public hatcheries refine protocols and apply them in the private sector. Commercial adoption will develop when cryopreserved sperm becomes more cost-effective than traditional spawning methods for individual species.

Marketing Considerations

In the analysis above, we discuss the basic costs associated with fish sperm cryopreservation on a generic level. Further estimation of these costs for a particular species requires additional information on broodstock collection and holding costs, sperm production rates, sperm-to-egg application ratios, fertilization rates for fresh vs. cryopreserved sperm and dilution ratios for extenders and cryoprotectants. Such parameters coupled with estimates of potential genetic gain for specific production traits will ultimately define the commercial feasibility of cryopreservation with aquatic species. However, species-specific analyses are time consuming and a more generalized approach may be useful for the preliminary identification of those applications where the technology poses the greatest commercial potential.

Recognizing Market Potential

Simple observations on the early d of cryopreservation in the dairy industry may provide insight on how and where markets for fish sperm might develop. For example, the advent of cryopreserved bull sperm occurred in a dairy industry that already had significant economic impact in the 1950's. For this industry to adopt cryopreserved bull semen, the applications had to be technically feasible – and it had to work well for people to use it. Finally, the use of cryopreserved dairy bull semen was probably facilitated because of the dairy industry's dependence on artificial insemination. Used collectively, these rudimentary observations form useful criteria for evaluating the marketing potential of cryopreserved sperm with aquatic species.

Eel, Catfish, or Salmon?

Consider the economic impact of three aquatic species: European eel *Anguila anguila*, channel catfish *Ictalurus punctatus* and Atlantic salmon *Salmo salar*. The European eel has an established market worldwide and is readily cultured in France, Italy and Spain. The channel catfish is the single largest aquaculture commodity by value in the U.S., and the Atlantic salmon is a highly prized market fish produced in Norway, British Colombia, Chile and elsewhere (Avault 1996). Using a checklist format (Table 3) we confirm that each of these species has considerable economic impact. However, technical feasibility of reproduction using cryopreserved sperm has only been documented for two of the species (channel catfish and Atlantic salmon). Furthermore, there is good reason to believe that industry utilization of cryopreserved sperm in the near future is only likely with one of these species, Atlantic salmon. We find support for this assertion by again considering the market development for cryopreserved dairy bull sperm.

Table 3. Criteria for marketing of cryopreserved fish sperm.

Critieria for marketing cryopreserved fish sperm	Species		
	European eel	**Channel catfish**	**Atlantic salmon**
Economic impact	Yes	Yes	Yes
Technical feasibility	No	Yes	Yes
Industry utilization	No	No	Maybe

From Artificial Insemination to Artificial Spawning

Would the technical feasibility of frozen sperm have been sufficient impetus for the dairy industry to begin utilizing cryopreservation in the 1950's? Obviously, the product had to work and do so cost-effectively. However, the pre-existing infrastructure of artificial insemination appears to have facilitated adoption because reproductive technicians in the dairy industry were already accustomed to collecting, transporting, storing and using sperm.

Consider again production of channel catfish. Despite the fact this industry has tremendous economic impact and reproduction with cryopreserved sperm is technically feasible (Tiersch et al. 1994), producers may be unwilling to utilize cryopreservation because of their reliance on an extensive pond-based spawning regime developed and refined over the past 60 yr. Switching to cryopreserved sperm of channel catfish would allow for some advantages, such as increased control in genetic selection programs; however, such a transition would mean a drastic departure from established methods of reproductive management, a remote prospect for this industry in the near future.

Conversely, Atlantic salmon producers may be more amenable to the prospect of cryopreservation. In this industry, shore-based hatcheries produce seedstock by manual stripping of gametes for fertilization and hatching. This human intervention in the reproductive process of fish is typically known as artificial spawning and it is analogous to the artificial insemination methods of the dairy industry prior to the advent of cryopreservation.

Compared to pond-based spawning regimes such as those in the channel catfish industry, adoption of cryopreserved sperm for use in established artificial spawning programs represents a less drastic technological shift for improving aquatic reproduction. If the dairy model provides any indication, the logical beginning for sperm cryopreservation is with those aquatic species currently produced by artificial spawning methods.

The Case of Hybrid Striped Bass Production

Hybrid striped bass of the genus *Morone*, represent aquaculture organisms with increasing economic impact where reproduction by cryopreserved sperm may be technically feasible. Furthermore, the production of hybrid striped bass (HSB) involves an artificial spawning regime that could benefit from cryopreservation if applications were proven to be cost-effective. A summary of this industry and a brief economic case study are provided to illustrate how commercialization of HSB research protocols might provide impetus for future markets in cryopreserved fish sperm.

Hybrid Striped Bass Background

United States production of HSB expanded over 900% in the 1990's, becoming one of the fastest growing segments of American aquaculture. Markets for HSB remain strong, but the industry is currently constrained by difficulties in the hatchery process. The hybridization of striped bass *Morone saxitilis* and white bass *Morone chrysops* is especially difficult because their spawning seasons only partially overlap and the fish are not always found in the same location. These constraints often limit hatchery production and can subject producers to shortages in seedstock. Kirby (1983, 1984) documented reproduction and growth of striped bass produced with cryopreserved sperm. More recently, additional research has been documented on the cryopreservation of sperm from striped bass and white bass (Brown and Brown, this volume).

Given the prospect of technical feasibility, it is plausible that cryopreserved sperm could yield economic benefits to HSB production. Such benefits might include a reduction in the temporal and spatial constraints of broodstock collection and spawning, expanding the time available for production of HSB seedstock and making hybridization more dependable while reducing hatchery costs. It is also worth noting that several state and federal hatcheries in the U.S. currently produce striped bass and hybrids for put-and-take fisheries. The availability of cryopreserved sperm could aid in reducing inbreeding in public hatchery stocks of striped bass and hybrids.

Setting up a Comparison

We have developed a hypothetical comparison for production of reciprocal hybrid striped bass production (female white bass x male striped bass) using fresh and cryopreserved sperm. Although production of reciprocal HSB requires additional steps (white bass eggs are adhesive and must be disaggregated before hatching), reciprocal hybrids are preferred by commercial producers because white bass females are typically easier to manage due to their smaller size and increased propensity for feeding in captivity (personal communication, Michael Freeze, Keo Fish Farm, Arkansas). Additionally, white bass eggs are small, having an egg number per unit spawn weight of ~ 4 times that of striped bass females (Harrell et al. 1990). These advantages are enhanced by the fact that reciprocal HSB production requires male striped bass, a fish known for producing copious amounts of sperm.

Table 4 lists general assumptions for comparing reciprocal HSB production using fresh and cryopreserved sperm. To begin, we arbitrarily set annual production at 80,000 Kg. Such a production level would require ~18 hectares of ponds assuming conservative stocking rates and an average harvest weight of 800 g per fish (100,000 fish at 2,000 Kg per hectares). With backwards extrapolation, we estimated the initial number of fry at 259,200, based on average mortality rates of 20% in Phase 3, 20% in Phase 2, and 80% in Phase 1. An estimate for the total number of fry does not necessarily translate to an equal number of eggs. Depending on ovulation rates, egg quality and handling, any particular spawn may be comprised of 0 to 100% of eggs that do not hatch. To account for this, we estimated egg demand at twice the total fry production. Therefore, based on 2,500 white bass eggs per g, a total of 250 g of eggs would be required for fry production (Harrell et al. 1990).

To determine the total volume of sperm required, we assumed a generous sperm-to-egg ratio of 1:10 (volume). For our case study, this ratio translates to a requirement of 25 mL of fresh sperm. Striped bass males used in HSB production typically average 2 to 5 Kg and a single large male could presumably supply all the sperm needed (50 mL). In this analysis we assumed that a minimum of ten males would be required to protect against mortality risks and to increase genetic variability. To estimate the amount of cryopreserved sperm we made some assumptions about its efficacy. Such estimates were difficult because sperm viability is only one of a variety of parameters that ultimately determine fertilization rates. For the sake of this study, we set the fertilization rate for cryopreserved sperm as 50% of the rate for fresh sperm. This translates to a doubling of the sperm requirement in the case for cryopreservation. We recognize that is a simple linear assumption and further analyses are required to address the economic requirements for additional eggs.

Table 4. Assumptions for comparison of fresh and cryopreserved sperm for production of reciprocal hybrid striped bass.

Annual production (Kg)	80,000
Area of farm (hectare)	18
Average harvest weight (g)	800
Total number of fish at harvest	100,000
Harvest weight (Kg/hectare)	2,000
Phase 3 mortality (%)	20
Phase 2 mortality (%)	20
Phase 1 mortality (%)	80
Total number of fry	259,200
Egg viability (%)	50
Number of eggs per g	2500
Total egg requirement (g)	250
Sperm:egg volume ratio	1:10
Total volume required for fresh sperm (mL)	25
Fertilization rate from frozen sperm (% of fresh sperm)	50
Total volume required for frozen sperm (mL)	50
Number of striped bass males	10
Sperm production per male (mL)	25
Total sperm collected (mL)	250
Sperm:extender volume ratio for cryopreservation	1:4
Cryopreservation straw size (mL)	5
Fresh sperm production units (useful/wasted)	25/225
Frozen sperm production units (useful/wasted)	125/0

Striped Bass Sperm: Fresh vs. Frozen

Returning to our chapter's initial question we now ask, *"how much does it cost to cryopreserve striped bass sperm?"* To answer this question, two things are needed: 1) an estimate of the generic costs required for fish sperm cryopreservation, and 2) a budget for broodstock collection of striped bass males. To begin, we return to Table 1 and consider a private cryopreservation program purchasing required equipment only. At the lowest level of storage capacity, total investment costs $9,497 or $1,900 per yr based on the 5-yr amortization schedule. Added to the yearly operating costs of $5,768, annual expenditures for the generic cryopreservation program would be $7,668 (Table 5).

Table 5. Annual costs for collection and storage of striped bass sperm (fresh and frozen) for production of reciprocal hybrid striped bass.

	Recruitment effort (# of trips)		
Item	**1**	**2**	**3**
Cryopreservation			
Fixed costs	$1,900	- -	- -
Operating costs	5,768	- -	- -
Subtotal	$7,668	- -	- -
Broodstock recruitment			
Guide fee ($250 per d)	250	500	750
Transportation (500 km @ $0.18/km)	90	180	270
Lodging ($75 per night)	75	150	225
Labor (2 people, 2 d, 8 hr per d, @$10 per hr)	320	640	1,280
Supplies	25	50	75
Total cost: fresh sperm	$760	$1,520	$2,600
Per unit cost (25 units)	$30	$61	$104
Total cost: frozen sperm	$8,428	- -	- -
Per unit cost (125 units)	$67	- -	- -

Secondly, we need to estimate the specific costs for the collection, transport and holding of striped bass males. Broodstock collection for HSB production is often coordinated with commercial guides, but recall that striped bass and white bass are not always available at the same time and location, therefore, costs for guided trips reflect only the costs for collecting striped bass males. Budget assumptions include a 500-km round trip at a $0.18 per km rate, a guide fee of $250 per d, $75 for lodging, $25 for supplies and $10 per hr for two employees working two 8-hr d (1 d fishing, 1 d transit). These costs are derived from similar expenditures incurred during our HSB hatchery and cryopreservation research at Louisiana State University.

Assuming all ten males are obtained in one trip, total costs associated for recruitment are $760 per yr. Adding this amount to the $7,668, costs for the cryopreserved striped bass sperm are estimated at $8,428 per yr. Compared to costs for fresh sperm, the increased costs of utilizing cryopreserved sperm may initially appear to negate any advantages associated with the technology. Yet, some additional considerations are required before we can realistically consider the cost-effectiveness of cryopreservation in this application.

The Hamburger Factor

The true advantages of fish sperm cryopreservation are illustrated in the obvious benefits of modern food refrigeration. For example, imagine having to slaughter a cow every time you wanted to eat a hamburger. Conversely, imagine having to justify the purchase of a freezer for the storage of only one hamburger patty. While these analogies may seem absurd, they serve to illustrate two important points. First, we must recognize that there is often a tremendous amount of sperm wasted during traditional methods of artificial spawning. In the case of striped bass, male fish frequently produce considerably more than the required amount of sperm. Secondly, it is not fair or realistic to evaluate the economic feasibility of fish sperm cryopreservation against

the costs of a single broodstock recruitment trip. Multiple recruitment efforts are often required in artificial spawning regimes for the production of aquatic species. Similar problems particular to the production of HSB include: 1) failure to obtain the needed number of males; 2) failure to harvest ripe males at an early date (white bass spawn on average about 1 mo earlier than striped bass) and, 3) failure to keep harvested males alive while waiting for female white bass to ovulate. A more realistic economic evaluation of cryopreserved sperm must account for the waste factor and the potential for multiple broodstock recruitment efforts.

Recall that only 25 mL of fresh sperm and 50 mL of frozen sperm was required to produce the needed amount of fish. However, a total of 250 mL of sperm would be available with a realistic collection rate of 25 mL from each of the 10 striped bass males. Thus, the "hamburger factor" is illustrated by a 25/225 ratio of useful/wasted production units under the traditional production scenario. Conversely, the ability to freeze sperm yields an extra 100 5-mL production units.

Expanding our comparison of fresh and cryopreserved sperm, we return to Table 5 and consider now the costs per production unit under a scenario of multiple recruitment trips. Recall that ten males were to be collected during a single collection trip. Under the cryopreservation scenario, annual costs are $8,428 or $67 per production unit. This cost is greater than $30 per unit for fresh sperm. However, a sec or third recruitment effort increases the costs of fresh sperm to $61 and $104 per unit. In general, the per-unit costs of fresh sperm increase as recruitment effort increases (Figure 4). On a per unit basis, cryopreservation yields 125 production units compared to the 25 units available using fresh sperm. The opportunity costs associated with wasted sperm become increasingly evident as additional attempts at artificial spawning efforts are required.

Additional attempts at fertilization are not uncommon for artificial spawning and may be necessary because of a variety of problems such as inferior egg quality, improper techniques or larval mortality. Additionally, sperm quantity and quality from captive broodstock tends to deteriorate over time and broodstock mortality is not uncommon because of the stress-related aspects of capture, handling and holding. These problems often result in the need for multiple broodstock recruitment efforts within and across production seasons. Thus, the hamburger analogy is repeatedly revisited.

Summary of Hybrid Striped Bass Case Study

Our comparison of fresh and frozen sperm has no shortage of assumptions and we realize that such budgets can be constructed in many different ways, with min changes in any one assumption providing different economic outcomes. However, the main purpose of this case study was not to develop discrete values, but rather to identify the primary economic variables needed for species-specific analysis and to illustrate the general relationships among parameters that affect economic feasibility. First, we assert that sperm cryopreservation for aquatic species is probably best suited for those applications in fisheries and aquaculture utilizing artificial spawning. In the case of HSB production, we specifically illustrate the need to compare the costs of frozen sperm under realistic consideration of the waste factor associated with artificial

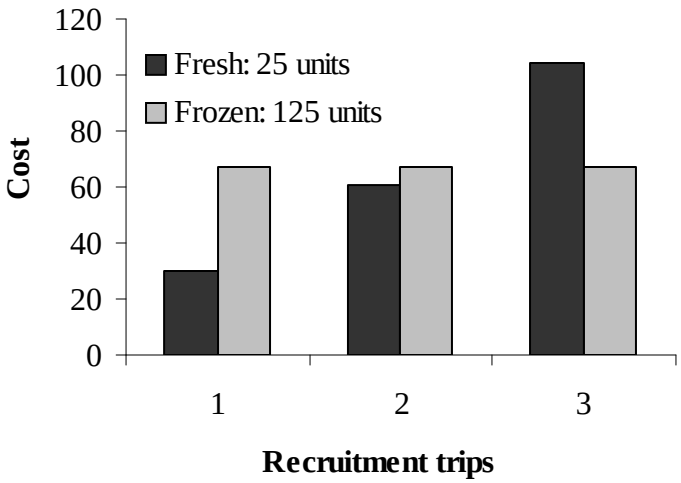

Figure 4. Cost per production unit for fresh and cryopreserved striped bass sperm.

spawning. Exactly how much sperm previously wasted could be collected and cryopreserved? The answer to this question will ultimately determine the number of available production units. In our analysis, 250 mL of striped bass translated to 125 cryopreserved productions units. However, this number could be higher or lower depending on variations in the total amount of collectible sperm, induced sperm production by use of hormones and variations in extender dilution rates.

Broodstock collection effort is perhaps one of the most critical aspects for determining the economic feasibility of cryopreserved fish sperm. In our case study we showed increasing feasibility for frozen sperm as collection efforts increased. However, the interpretation of collection is not limited to a single producer making one or more trips within a single yr. Additional interpretations might include a single producer with multiple recruitment trips across several yr or multiple producers each requiring sperm within and across spawning seasons. Evaluating these alternative economic scenarios will reveal additional information on the economics of cryopreservation applications.

Future Considerations

Cooperatives

Despite the potential economic feasibility of fish sperm cryopreservation, the immediate costs associated with investment in the technology may be prohibitive for a single investor. Such cost constraints were encountered in the early d of the dairy industry when AI emerged as an economic reaction to the prohibitive costs of maintaining bulls.

Business structures such as partnerships and corporations are traditionally used for spreading out the costs and risks of commercial investments. Among the many types of business structures available for marketing of fish sperm, cooperatives may represent the most logical alternative. The unique advantages offered by cooperatives are inherent to their definition. A cooperative is typically defined as:

> "…a business voluntarily organized, operating at cost, which is owned, capitalized, and controlled by member patrons as users, sharing risks and benefits proportional to their participation" (Roy 1981).

By the late 1800's, U.S. dairy farmers were forming crude cooperatives known as "breeders clubs" (Herman 1981). These organizations were initially no more than small groups of dairymen who collectively supported the purchase and maintenance of bulls. This centralization assured dairymen would have the needed services of quality sires at reduced cost. Eventually the cooperative nature of these organizations facilitated the adoption of technological innovations such as artificial insemination in the early 1900's and cryopreservation in the 1950's. Today, many of these organizations have evolved into super-cooperatives that specialize in harnessing commercially desirable genetic gain by utilizing innovative technologies such as gamete cryopreservation and embryo transfer.

Cooperatives offer similar advantages for market development with cryopreserved fish sperm. Returning to the HSB example, the investment and operating costs for cryopreservation may be more affordable if spread over several commercial cooperative members whose risks and benefits are relative to their level of participation. Furthermore, a state-run cryopreservation cooperative for fish sperm could offer additional advantages of a lower cost structure and increased technical expertise. Such public cooperatives were quite successful in the formative d of AI and sperm cryopreservation in the dairy industry.

Non-Market Species

The focus of this chapter has been to identify constraints and solutions related to the economics and marketing of cryopreserved fish sperm in fisheries and aquaculture. However, fisheries and aquaculture constitute only half of the applications discussed in this text. The use of cryopreservation for sperm and embryo-based conservation of threatened and endangered (T & E) species is an area with considerable economic and market implications as well.

Non-market valuation techniques have emerged in recent yr as the result of a paradigm shift in traditional economics. The shift occurred as increasing evidence mounted that neoclassical economic theory was insufficient for determining the value of non-exclusive and non-renewable resources (Costanza et al. 1997). A new branch of the discipline, environmental economics, utilizes non-market techniques for estimating externality costs (e.g. the cost of environmental pollution) and contingent, in-situ, and bequest values (e.g. the value of an endangered species). An environmental economics approach has already been applied in the area of T & E species by way of a contingent valuation method that estimates public willingness-to-pay (WTP) for conservation of a particular species (Walsh et al. 1984). Similar potential exists for deriving new WTP estimates of the value of cryopreserved gametes and embryos of T & E species. Such data might prove useful for validating the efforts of government fish hatcheries conducting cryopreservation-based conservation efforts or developing germplasm repositories. Eventually, market-based incentives for conservation could emerge in which direct or indirect payments are made by governments to commercial entities for cryopreservation of T & E species. Such market-based incentives have gained momentum in recent yr due in large part to the failure of regulatory approaches to control the loss of natural resources from the ecosystem to species levels.

Conclusions

We conclude by reiterating some of the critical points we have outlined in this chapter (Table 6). Perhaps the most astounding point is that after 40 yr of research into fish sperm cryopreservation, there is only one documented report pertaining to the economic aspects of this technology (Caffey and Tiersch 1999). Any possibility of commercialization hinges on knowledge of the basic costs associated with application in aquatic species. The specificity of this economic information must increase for cryopreservation to advance beyond research and into commercial application with aquatic species. Preliminary generic cost analysis indicates that public hatcheries currently hold an advantage over private hatcheries trying to establish cryopreservation programs. Lower cost structure and increased insulation from economic risks could translate to a greater potential for cryopreservation investments being made at public hatcheries. Technology transfer by these facilities to the private sector would aid development and refinement of commercial protocols. Adoption of these protocols will require cost-effectiveness compared to traditional spawning methods. However, the technical feasibility of reproduction using cryopreserved fish sperm is commercially irrelevant in species without significant economic impact. Furthermore, it is most likely that commercial utilization of cryopreservation will occur in those aquatic species produced by artificial spawning.

Table 6. Points for discussion regarding the economics and marketing of cryopreserved fish sperm.

Economic information is crucial for determining the feasibility of any application of cryopreservation.
Public fish hatcheries have lower barriers to cryopreservation investment because of their lower cost structure and existing technical expertise.
Market development hinges on refining the technical feasibility of reproduction via cryopreservation.
Technical feasibility of cryopreserved sperm is commercially irrelevant in species with little or no economic impact.
Artificial spawning is analogous to artificial insemination and constitutes a major requirement for commercial adoption of cryopreserved sperm in aquatic species.
Economic comparisons of fresh and frozen sperm should incorporate the opportunity costs of wasted sperm.
Economic comparisons of fresh and frozen sperm should incorporate the possibility of multiple recruitment efforts within and across production facilities and spawning seasons.
Cooperatives represent the most logical business structure for market development with cryopreserved fish sperm.
Public fish sperm cooperatives will initially offer the greater advantages for industry development with aquatic species.
There is much potential for environmental economists to evaluate the costs and values of cryopreservation-based conservation with threatened and endangered species.

A case study with HSB illustrates that the economic feasibility of fish sperm cryopreservation is ultimately a function of reducing waste and effort. How much previously wasted sperm can be cryopreserved? How can cryopreservation reduce the costs and risks associated with broodstock recruitment? What are the commercially relevant genetic traits of the culture species and to what extent can cryopreservation help us to capitalize on their heritability? As seen with the dairy industry, commercial development of markets for fish sperm would probably be enhanced by formation of producer cooperatives. Such organizations have facilitated the use of cryopreserved bull semen in the dairy industry by spreading out costs and risks and by reducing the technological constraints associated with cryopreservation.

Finally, this chapter has focused primarily on commercial applications of fish sperm cryopreservation for fisheries management and aquaculture; however, the use of this technology for conservation of T & E species is an area where environmental economics has much to contribute. Application of alternative economic valuation approaches such as WTP, may eventually be necessary for providing the economic values associated with non-market aquatic species and the justification for cryopreservation-based conservation efforts.

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Annotated Bibliography of Developments in the Last Decade

Although industrial aquaculture has been developing for decades throughout the world, few investigations have specifically focused on the economics and marketing of cryopreserved gametes within aquatic organisms. This chapter focused on cost and return scenarios. Subsequent studies have addressed additional facets, including the willingness to adopt cryopreservation technologies in existing hatcheries and the economics of sex selection regimes. Despite these advances, commercialization remains in the infant stages, and additional analysis and research will be needed to demonstrate potential industry applications within aquatic species.

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New Chapter

Willingness-to-Pay for Specific Genetic Improvements for Aquaculture Species

Brian P. Boever, R. Wes Harrison and Terrence R. Tiersch

Introduction

Whether consumers in the United States know it or not, approximately 30% of their seafood is being raised through aquaculture production (Harvey 2003). Aquaculture in the United Staes primarily consists of production of foodfish, ornamental fish, baitfish, mollusks, crustaceans, aquatic plants, and reptiles such as alligators and turtles (ERS 2003). Rapid growth has occurred within many aquaculture industries since the 1980s, resulting in a quadrupling of U.S. aquaculture production during that time. In 2005 the total value of U.S. aquaculture production was approximately $1.09 billion (2005 U.S. Census of Aquaculture). Foodfish production, which included catfish, trout, salmon, tilapia, hybrid striped bass, sturgeon, yellow perch, and walleye, made up approximately 60% of total sales (2005 U.S. Census of Aquaculture).

Despite the growth in U.S. aquaculture, a variety of challenges have emerged, for example, in production of channel catfish *Ictalurus punctatus* which is an important regional and national market. In 2007, nearly 500 million pounds were processed in the southeastern U.S., and prices paid to farmers ranged from $0.65 to $0.83 per pound (USDA NASS 2008). In the past few years, however, the industry has experienced a catastrophic reduction and continues to face severe challenges. Between 2001 and 2007 the number of fingerlings produced by the four major states (Mississippi, Arkansas, Alabama, and Louisiana) shrank by approximately 22% (USDA NASS 2008), and approximately 40,000 water-acres were taken out of production. The decrease in production is attributable to reduced profit margins from low prices paid to farmers, increased costs of fuel and feed ingredients, and increased foreign competition. Asian countries have become the predominant suppliers in global aquaculture markets, with China alone accounting for over 70% of the total volume of world aquaculture production, and close to 50% of the total world value (FAO 2004). Less expensive labor and an abundance of land suitable for aquaculture production has led to the emergence of global competition. Consequently, a major challenge for U.S. aquaculture is to remain competitive in domestic and global markets. One way to accomplish that is to develop and adopt new technologies and production systems that allow lower production costs. One approach with essentially unexploited potential to increase efficiency is to improve the genetic attributes of fish stocks. Greater control over genetics will allow producers to improve product consistency and lower production costs.

Accordingly, we sought to quantify the awareness and perceived value of the kinds of genetic improvement that could be made available through commercial-scale use of cryopreservation and development of markets for genetic resources within U.S. aquaculture. We employed a choice-based conjoint analysis to determine the importance of genetic improvements to grow-out producers and estimated willingness-to-pay for selected attributes.

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Cryopreservation and Genetics

The ability to selectively breed fish is a proven route to more profitable outcomes. The most obvious potential profits would be from improved fish stocks, resulting from the use of selected males. Some fish characteristics that may be deemed desirable include: faster growth, higher dressing percentage (more meat and less waste), greater feed efficiency, increased resistance to disease, and higher tolerance to poor water quality and stressful conditions (Avault 2002). The use of selective breeding leads to controlling the genetics of populations.

The availability of cryopreservation services and frozen germplasm from selected broodstock would enable farmers to more easily access and control improvements in genetic attributes desired in their product line. For instance, if breeders select for broodstock that can survive a fatal disease, the sperm of those fish could be frozen and used for producing disease-resistant offspring. Hatchery operators would have access to and could utilize the genetic makeup of particular fish, or groups of fish, for multiple years. Also, cryopreservation offers the most efficient way to control genetics. Currently it takes years of breeding to establish defined genetic lines, and this process is greatly complicated by the constraints in making necessary crosses and maintaining broodstock populations across years for comparisons. Cryopreservation can increase capabilities for fully crossed breeding designs and allows genetic screening of males by molecular markers for breeding studies. There are inherent, structural differences across aquaculture industries that influence the application of cryopreservation (e.g., Caffey and Tiersch 2000a). For example, presently the species with the most prevalent genetic control is Atlantic salmon *Salmo salar*. Because domesticated broodstocks are used, specific lines are well established and are well suited for application of cryopreservation for genetic improvement. On the other hand, the U.S. hybrid striped bass industry (based on hybrids within the genus *Morone*) has essentially no genetic control because of reliance on wild-caught broodstock each year to artificially spawn within the hatchery. As such, this industry would first rely on cryopreservation simply to ensure that sufficient volumes of sperm were available for annual production of seedstock (i.e., avoiding the potential opportunity costs of collecting too few broodstock).

Genetic Improvement

For genetic improvement to occur through selective breeding there must be a link between the observed attributes a fish displays and its genetics. The attributes a fish displays (phenotype) are determined by environmental influences and genetic influences. One major genetic influence is additive genetic variance (heritability), which refers to the correlation an individual parental phenotype has with phenotypes of offspring, and is measured as the percentage of total phenotypic variation for the trait in question. For genetic improvement to be accomplished using selective breeding, there must be a significant level of heritability associated with a specific trait. Low heritability implies that little genetic improvement can be gained from one generation to the next with the use of selective breeding because there are too many other contributing factors to the phenotype of the offspring (Lutz 2001). However, even with high heritability, a fish stock that is bred to grow faster may not because the environment does not allow for such growth. If the stocking rate is too high or the water quality too low, the stock will not grow beyond what the environment allows.

Numerous studies have focused on determining the heritability of specific attributes for specific species. In one study, the average heritability of body weight represented 34% of the phenotypic variation for 3 strains of channel catfish (Dunham and Smitherman 1983). High

heritability percentages were shown for weight as well as length in channel catfish (Reagan et al. 1976). Heritability estimates for disease resistance have also been studied. One study found that selective breeding would be practical and effective in Atlantic salmon to increase resistance to furunculosis because heritability for the trait was around 40% (Gjedrem et al. 1991). Selective breeding in Nile tilapia *Oreochromis niloticus* found a relatively high heritability for body weight at 16 wk with an average gain of 12% per generation (Bolivar and Newkirk 2002). The influence of heritability on phenotypic traits varies among species and within a species, but selective breeding is a proven approach to attain genetically improved fish stocks (Lutz 2001), and can be routinely captured and exploited by use of cryopreserved germplasm.

New Products and Technologies and Early Adopters

Opening new approaches to genetically improved fish stocks can be viewed as a new technology adoption problem. Early adopters of a new technology face uncertainty about the effects a new production system, or improved input, will have on costs and profitability. In general, adoption of new technologies entails some investment in equipment or the purchase of more expensive inputs. Hence, cost saving are only possible if the new technology provides greater output per unit of input, or through improved outputs that may be sold at higher prices. Early adopters face uncertainty regarding how effective the new technology will be in lowering costs, as well as whether an improved output will bring a premium price in the market. Studies show that factors influencing whether an operator is an early adopter include the manger's level of education, various firm-specific characteristics, and the manager's knowledge about the new technology (Olmstead and Rhode 1995, Wozniak 1987, Wozniak 1993). Firm-specific characteristics include the level of debt, dependence on off-farm income, and the operational scale of production. Knowledge about the new technology is the amount of information known by the manager regarding the product or service being offered. The ability to apply new technologies depends on the manager's capacity to identify sources of information, and to process and decode relevant information. Studies show that higher levels of education and available information about the product in question reduce the costs and uncertainty of adoption, and therefore increase the likelihood of early adoption. In general producers with larger scales of production are more likely to be early adopters than producers with smaller scales of production.

Little is known about which attributes are preferred by aquaculture grow-out producers in the U.S., or how much producers are willing to pay for them. The genetic attributes examined in this study were growth rate, disease resistance, and resistance to low dissolved oxygen levels. The objective of this study was to measure the willingness of U.S. hatchery and grow-out producers to adopt and pay for genetic improvement in fish stocks. A conditional logit model was used to estimate willingness to pay (WTP) for selected genetic attributes for the selected species. We report results from a national survey of producers of channel catfish (and hybrids with male blue catfish *Ictalurus furcatus*), hybrid striped bass, tilapia, Atlantic salmon and rainbow trout *Oncorhynchus mykiss* in the United States. This study is the first of its kind to evaluate the preferences for genetic attributes from producers. It also is the first known study to evaluate preferences for cryopreservation services by the producers in aquaculture industries.

Methodology

Theory Behind Willingness-to-Pay Determinations for Producers

A producer's willingness to pay (WTP) can be examined in the context of profit maximization subject to a given production technology. Assume that a firm is considering a change from one quality of input to another (i.e., q_0 to q_1). The WTP associated with the change can be modeled as:

$$WTP = \Pi_1(p, w, \mid q_1) - \Pi_0(p, w, \mid q_0)$$

where w is a vector of input prices, p is a vector of output prices, and q is a given quality of a primary input in production. This yields the indirect restricted profit function, $\Pi(p, w, \mid q)$, where $\Pi_0(p, w, \mid q_0)$ is the indirect profits given an input quality of q_0. The WTP represents the change in profit the producer expects to receive by acquiring q_1 instead of q_0 (Lusk and Hudson 2004).

In the context of this study, the initial profit $\Pi_0(p, w, \mid q_0)$, consists of input prices (w) that grow-out producers are paying for conventional fingerlings. The adoption of genetically improved fingerlings will affect $\Pi_0(p, w, \mid q_0)$ through improved fingerlings (q_1) that are expected to increase the production efficiency of the operation. This may be through improved growth rate or enhanced disease resistance. Improved efficiency would presumably reduce costs, thereby leading to higher profits and a positive WTP for fingerlings. The difference between Π_1 and Π_0 represents the producers WTP for improved fingerlings.

Review of Related Studies

Most economic studies regarding aquaculture have dealt with evaluating the production feasibility of a species, determining the cost-effectiveness of a new system, or reviewing a particular policy implication. For instance, the adoption of flow-through and recirculating technology in soft-shelled crab production was studied based on the characteristics of the producer (Caffey and Kazmierczak 1994). Also studied were the production costs incurred by a farm or institution that incorporated cryopreservation into its existing operation (Caffey and Tiersch 2000b). The effects on a particular industry (such as salmon) stemming from government regulations and their influence on market structure have been studied (Tveteras 2002).

Other studies have focused on consumer preferences for seafood attributes (e.g., Holland and Wessells 1998, Anderson 2000). For example, a study of the market for farm-raised hybrid striped bass determined that price and product form were the attributes most important to mid-Atlantic seafood buyers (Halbrendt et al.1991). However, attributes that the consumer values is likely to be different from the attributes that a grow-out farmer would value. Consumers are concerned with features such as price, serving size, product form, how the product was obtained (farmed or wild-caught), color, or presence of ecological labeling (Wessells 2002). Producers are more concerned with growing the stock as economically efficient as possible. No research has been directed towards the valuation of specific genetic attributes of aquatic species by producers.

Stated Choice Analysis

Stated choice analysis, also known as discrete choice, contingent-choice, choice experiments, or choice-based conjoint analysis (CA), is a type of analysis in which hypothetical products are evaluated by a subject. Stated choice techniques are a means to evaluate the potential market for a new product, or to identify the most important attributes of an existing product (Lee et al. 2000). These techniques enable researchers to evaluate market situations that do not yet exist. In a choice-based conjoint experiment, respondents are asked to choose a

preferred alternative from a set of alternatives, rather than ranking or rating alternatives, which is a more typical CA format (Adamowicz et al. 1998). Several researchers have found discrete choice methods to be superior relative to the ranking or rating technique (Pinnell 1994).

In stated choice analysis, respondents are only allowed to choose one option per choice-set. The researcher determines the number of alternatives per choice set, which are typically limited to between two and four. The inclusion of an opt-out, or "neither," option is also common. This serves as an "opt-out" base and is available to all respondents. The stated choice method was chosen for this study because it mimics real market situations better than ranking or rating. In a market situation, individuals are faced with the choice of purchasing one product over another, or not purchasing either. While the ranking or rating methods allow for more responses per respondent, the reliability of the information is questionable for specific situations. Certain options would never be chosen in a real market environment. There is no real way to establish which options would never be chosen if there was no inclusion of an opt-out response. Also, response bias and respondent fatigue increase as the number of alternatives increase (Louviere et al. 2000). The stated choice methods are in line with random utility theory and can be analyzed with random utility models, unlike ranking/rating conjoint methods (Louviere 1994).

Fish Stock Attributes for This Study

Pre-testing of survey design and attribute selection were completed using the assistance of aquaculture extension agents and farm operators. The attributes selected for this study should be representative of the various aquaculture species that make up the foodfish sector. Also, there was a need to keep the amount of attributes to a minimum, so that the resulting choice scenarios would minimize respondent fatigue. The four attributes used in the final version of the survey were growth rate, disease resistance, resistance to 10% lower dissolved oxygen levels, and price. All these attributes are important in the production of any species. They also have important economic implications for the grow-out operation. Faster growth rates mean shorter production cycles and improved feed efficiency. If fewer fish die due to disease outbreaks then production efficiency will increase. A higher tolerance to less than desirable oxygen levels means lower costs associated with monitoring and regulating oxygen levels, as well as higher survival rates in low oxygen conditions.

Each attribute was expressed in terms of either two or three levels. Growth rate and disease resistance were expressed as being either at their current level (that is, no change from the operator's current growth rate or disease resistance), or a 10% improvement in growth rate and disease resistance relative to the current level, or a 20% improvement relative to the current levels. If a producer currently averages a loss of 200 fish per production cycle, a 10% increase in disease resistance would result in an average loss of only 180 fish. The attribute "resistance to 10% lower dissolved oxygen levels" refers to the ability of fingerlings to tolerate 10% lower levels of dissolved oxygen in the water without dying. This attribute was expressed as either a "yes", implying the fingerling possesses an ability to survive in 10% lower oxygen relative to conventional oxygen levels, or "current", which implies the fingerling can survive in conventional oxygen ranges. The price attribute was expressed as a price premium, an amount that producers would be willing to pay above current fingerling price (20%, 40%, and 60%).

Choice Task Design

There are numerous ways to prepare a stated choice questionnaire. This study elected to utilize the no-purchase or "neither" alternative (i.e., prefer status quo). With the inclusion of a

"neither" option, respondents had the opportunity to pay a zero price premium because they could choose a non-genetically improved fish stock. Along with the "neither" option, respondents were presented with a pair of alternatives, each with at least one genetically improved attribute. The four attributes chosen (with 3 x 3 x 2 x 3 levels), resulted in 54 possible product combinations. However, this number was too high to realistically be completed without causing respondent fatigue (or a reduction in overall response rate). Therefore the software package Bretton-Clark Conjoint Designer was used to formulate 9 orthogonal attribute combinations. Three more product combinations were added to the design to provide a balanced number of choice tasks. This resulted in twelve alternatives for genetically improved fish stocks to be evaluated by U.S. grow-out producers. Six choice sets were constructed, where each included two of the twelve genetically improved options. The first and second alternatives were paired to form the first choice set, the third and fourth alternatives were paired to form the second choice set, and so on until all six choice sets were formed. Because of the length of the overall questionnaire, a split-sample approach was used. Three versions of the questionnaire were mailed to aquaculture producers, with each version having two choice sets to evaluate. Each version of the questionnaire was randomly assigned to a producer in the mailing list, which included 1,293 U.S. aquaculture farms. Respondents were asked to select their preferred option in each set. An example of a choice task is included in the Appendix.

Survey and Data

The survey was administered during the summer of 2005. Usable responses were returned from 11.8% of the total surveys mailed. In addition to the previously described choice task, the survey also elicited information regarding preferences, beliefs, and opinions of aquaculture producers across the U.S. about topics such as cryopreservation, genetic improvement, and the future of aquaculture. These responses could be used to determine which issues were most important to the various groups and segments of aquaculture producers. Questions concerning prior knowledge about cryopreserved sperm were included in the questionnaire to determine whether that firm was a potential early adopter. Firm-specific questions such as farm size and gross sales, individual-specific questions (i.e., education level of the farm manager), as well as questions specifically asking managers about their history and potential likelihood of adopting a new technology were also included in the survey. The survey was divided into three sections. The first applied only to farms that participated in spawning activities. The second, applied only to farms with grow-out operations. The third section applied to all aquaculture farms and included mostly demographic information. The stated choice questions were included only in the grow-out section of the questionnaire.

Model Formulation

Choice-based modeling is derived from random utility theory, which assumes that consumers maximize their utility with the choices that they make (Louviere et al. 2000). In the present study, we assume that maximizing profits for their operation is analogous to maximizing the operator's utility with respect to factors that affect firm level profits. Because researchers have incomplete information regarding the characteristics that make up the decision process, the random utility model separates total utility into two parts. The first is a deterministic component, (V_{ij}) and the second is a stochastic, or random, error component (ε_{ij}) (McFadden 1974, Louviere et al. 2000, Heiss 2002). The resulting utility equation is:

$$U_{ij} = V_{ij} + \varepsilon_{ij}$$

where U_{ij} is the utility of the i^{th} consumer choosing the j^{th} product. Individual i will choose product j only if $U_{ij} > U_{ik}$, where k represents an alternative product. The probability that individual i will choose alternative j out of a set of k alternatives for all k in the choice set not equal to j is:

$$\Pr_{ij} = \Pr\left(V_{ij} + \varepsilon_{ij} \geq V_{ik} + \varepsilon_{ik}; \forall k \neq j\right)$$

The conditional logit (CL), multinomial logit (MNL), and nested logit (NL) models are common methods used to analyze discrete choice variables. The nested logit model relaxes the independence of irrelevant alternatives (IIA) assumption. The IIA implies that the ratio of choice probabilities, for choosing one alternative over another, is not affected by adding or omitting additional alternatives. The MNL and CL do not relax this assumption. The MNL utilizes individual specific explanatory variables, whereas the CL model focuses on the characteristics of the alternatives for each individual and uses them as explanatory variables. The difference between the two models is shown in the following equations:

$$\text{MNL:} \qquad P_{ij} = 1 / \sum_{k=1}^{J} \exp\left[X_i\left(\beta_k - \beta_j\right)\right]$$

$$\text{CL:} \qquad P_{ij} = 1 / \sum_{k=1}^{J} \exp\left[\left(Z_{ik} - Z_{ij}\right)\alpha\right]$$

where X_i is the individual specific characteristics of individual i, β and α are the parameter vectors, and Z_{ij} represents the characteristics of the j^{th} alternative for i individual. The probability in the MNL model is subject to the difference in coefficients for the alternatives. However, the probability of the CL model depends on the difference in the value of the characteristics across alternatives (Hoffman and Duncan 1988). The CL allows explanatory variables to differ among choice options, and allows analysis of the attributes in the alternatives as opposed to analyzing the attributes of the individual selecting the alternative (Jepsen and Jepsen 2002).

The conditional logit model with interactions (CLI), or mixed logit model, is a hybrid of the MNL and CL models. The CLI model allows preferences to be heterogeneous by incorporating individual-specific characteristics into the model as interaction terms. Preference heterogeneity allows preferences to vary among individuals. This means that the impact on utility from changes in fish stock characteristics can vary across grow-out producers either randomly or logically (Breffle and Morey 2000, Birol et al. 2005). It is reasonable to assume that the characteristics of the operator, as well as the operation, will have a significant impact on the preferences for selecting fish stock alternatives. The individual-specific (or operator-specific) characteristics may include socioeconomic characteristics as well as variables representing the respondent's attitude towards a certain subject.

The CLI utilizes individual-specific explanatory variables, which are estimated in a MNL, as well as the alternative-specific variables, which are estimated in the normal CL model, to form a mixed model. The model is as follows:

$$\text{CLI:} \qquad P_{ij} = \sum_{k=1}^{J} \exp\left(X_i\beta_j + Z_{ij}\beta\right) / \exp\left(X_i\beta_k + Z_{ik}\alpha\right)$$

where X_i is the individual specific characteristics of individual i, β and α are the parameter vectors, and Z_{ij} represents the characteristics of the j^{th} alternative for i individual (Hoffman and Duncan 1988). The CLI allows explanatory variables to differ among choice options. The CLI model not only allows for the analysis of the attributes in the alternatives, but it also allows

identification of which attributes of the individual affect choice selection (Jepsen and Jepsen 2002).

The CLI model was applied to the data in the choice-based portion of the questionnaire. The CLI model maintains the assumption of independent and identically distributed (i.i.d.) error terms. There is a precedent for this in previous studies. For example, analysis of the attributes contributing to the utility of a moose hunting trip included the addition of interaction terms to the normal CL model to evaluate the impact of being from an urban area on the choice selection of a hunting site (Adamowicz et al. 1998). Another study evaluated the socioeconomic impacts on the selection of a forest design (Hanleyet al. 1998). In a study that evaluated the attributes associated with a wetland management program, interaction effects were measured for various individual-specific variables (Birol, et al. 2005). The practice of using interaction terms can allow results to be individual specific.

Results

Seventy respondents from the survey reported that they conducted grow-out operations (Table 1). Twenty two respondents (32%) indicated that channel catfish is their primary product and 20 (29%) of respondents indicated that rainbow trout is their primary product. Fifty one of the respondents (74%) reported that they grow only one species; with the remaining 19 (26%) indicating they produce multiple products. Hybrid strip bass producers accounted for 12% of the respondents, followed by producers of tilapia, which accounted for 10% of the total. Atlantic salmon producers represented only 3% of the total.

Table 1. Species distribution of grow-out operations.

	Primary Product [a]	Percent	Only Product [b]	Percent
Channel catfish	22	32	18	35
Hybrid striped bass	8	12	2	4
Tilapia	7	10	7	14
Atlantic salmon	2	3	2	4
Rainbow trout	20	29	13	25
Other	10	14	9	18
Totals	70	100	51	100
Percent of farms with only one product = 74%				
Percent of farms with multiple products = 26%				

a. Primary product is defined as the species with the highest reported percentage of sales.
b. Respondents who reported the species is the only one they produce.

An overwhelming majority of grow-out farms reported they are privately held company and employ less than 10 workers (Table 2, next page). More than 50% of the respondents reported that they used ponds as there production system. This is expected due to the high number of catfish farmers. Over 40% used flow-through systems, which is attributed to the trout farmers. The highest category for sales is $250,000 to $1 million. Only 4.7% of the respondents generated more that $5 million in sales. Most of the respondents had some college education or graduated from college with a bachelor's degree or an advanced degree. More than 60% of the respondents were 46 yr of age or older.

Table 2: Summary statistics for respondents with grow-out operations

Production Method	#	%	*Ownership*	#	%
Pond	36	51.4	Public	5	7.1
Flow-through	29	41.4	Private	64	91.4
Net pens/cages	3	4.3	Both	1	1.4
Closed recirculation	14	20.0	Total	70	100
Total	70	100			
Employees	#	%	*Sales (x $1,000)*	#	%
Less than 10	55	78.6	Less than 2.5	3	4.7
10 to 50	13	18.6	2.5 to 9.999	3	4.7
51 to 150	2	2.9	10 to 49.999	8	12.5
More than 150	0	0	50 to 249.999	15	23.4
Total	70		250 to 999.999	21	32.8
			1,000 to 4,999.999	11	17.2
			More than 5,000	3	4.7
			Total	64	
Education	#	%	*Age (yr)*	#	%
Less than high school	1	1.4	18 to 24	1	1.5
High School graduate	8	11.4	26 to 35	5	7.3
Some college	19	27.1	36 to 45	18	26.1
Bachelor's degree	25	35.7	46 to 60	38	55.1
Advanced degree	17	24.3	Older than 60	7	10.1
Total	70	100	Total	69	100

Conditional Logit with Interactions

Results for the mixed logit model are presented in Table 3 (next page). The overall model was found to be significant at the $\alpha = 0.01$ significance level with a log likelihood ratio value of 64.20. The price premium variable was coded as 0, 20%, 40%, or 60% for the available price premiums above current fingerling prices. The rest of the variables were effects coded in the data set and the "neither" option served as the base and was coded as 0 throughout. The alternative-specific constant (ASC) "ab," which represented the genetically improved alternatives, was held out of this model. This was done to reduce the chance of multicollinearity, because all individual-specific variables were interacted with the ASC term. Interactions were included in this model to determine which individual-specific variables significantly affected the selection of a genetically improved alternative.

The signs of most of the alternative-specific coefficients were as expected, negative for the price premium and positive for genetically improved attribute levels. The coefficient

Table 3. Conditional logit with interactions, payment and Willingness-to-Pay (WTP) estimates

	Coefficient	St. Error	WTP	R.I.[a]
Growth Rate Attribute				40.53
10% Increase	.567**	.283	17.61	
20% Increase	.695***	.242	21.58	
Disease Resistance				17.19
10% Increase	.366	.264	11.35	
20% Increase	.099	.307	3.08	
Tolerance to Low Dissolved Oxygen	-.055	.163	-1.69	2.26
Price premium	-.032**	.013		40.01
Firm- Specific Interactions				
Hatch*ab	.291	.286		
Channel catfish*ab	1.399***	.503		
Tilapia*ab	1.361**	.619		
Rainbow trout*ab	.632	.432		
Atlantic salmon*ab	.123	.838		
Hybrid striped bass*ab	-.790	.571		
Private*ab	6.209***	2.139		
Sales greater than 50K*ab	.264	.311		
Operator-Specific Interactions				
Bachelor's degree*ab	.964***	.329		
Multiple new technologies in last 5 yr*ab	1.385***	.418		
36-60 yr of age*ab	-3.144**	1.392		
Over 60 yr of age*ab	7.215**	3.112		
Favorable toward Cryo*ab	.341	.323		

N = 324 ; LR(19) = 64.20***; Pseudo R^2 = .2705.
*, **, ***. Denotes Significance levels at α= .10, .05, and .01 percent, respectively.
a. Relative importance (RI) measures the percentage of the selected attribute's part-worth range to the sum of part-worth ranges for all attributes.

associated with resistance to low dissolved oxygen levels was negative but not statistically significant. The results show that growth rate and price premium were the most important genetic attributes for grow-out producers, tied with relative importance factors of 40%. The levels of growth-rate and the price premium were the only significant genetic attributes in model. Moreover, producers were more likely to choose an alternative with 20% increased growth rate than an option offering only a 10% increase.

Firm-specific variables were included as interactions in the model. The firm-specific variables were effects coded (1,-1). All firm-specific variables were placed in interaction with the alternative-specific constant (ab) to analyze the interest for genetic improvement by specific types of farms. For example, the variable "Private*ab" represents privately run grow-out farms that selected either of the two genetically improved options. The positive and significant coefficient associated with variable indicating private grow-out farms (Private*ab) implied that private farms are more likely to adopt genetically improved fish stocks relative to publically managed operations. All species interactions were positive except for the hybrid striped bass interaction variable. However, only the variable indicating the operation produced predominantly catfish and tilapia was statistically significant, which implied that catfish and tilapia producers were more likely to adopt genetically improved stock relative to producers of other species.

Atlantic salmon, hybrid striped bass, and rainbow trout interactions were not significant in this model.

Operators-specific variables were also included as interactions in the model, and were coded using conventional 0,1 dummy variable coding. Respondents having incorporated multiple new technologies in the last 5 yr, as well as managers having a Bachelor's degree, each had a positive and significant effect on choosing a genetically improved alternative. Two of the three age variables were included in this model. Surprisingly, respondents over the age of 60 yr had a strong positive and significant coefficient, indicating a higher likelihood of adopting a genetically improved fingerling relative to the omitted category of 18 to 36 yr. However, operators between the ages of 36 and 60 years of age were less likely to select the improved alternatives relative to the youngest operator category. The variable "Favorable toward Cryo*ab" represents the operator's attitude toward cryopreservation, but it was not significant in the model. Also found not to be significant was the variable "Sales greater than 50K*ab," which represented farms that grossed more than $50,000 in the previous year. This meant the relative size of the operation had no significant effect on the willingness to adopt a genetically improved fish stock.

The willingness-to-pay for attribute *i* was calculated as the negative ratio of the coefficient for attribute *i* and the price premium coefficient. It was calculated as:

$$WTP_i = -\frac{\beta_i}{\alpha}$$

where β_i is the coefficient of attribute *i* and α is the price premium coefficient. The willingness-to-pay values are interpreted as the percentage premium above current prices that producers are willing to pay to obtain a fingerling with the specific genetic attribute. Results of this model showed that grow-out producers were willing to pay approximately 18% more for fish stocks with a 10% increase in growth rate. Producers would pay about 22% more per fingerling for a 20% increase in the growth rate of their stocks.

Conclusions

A nationwide survey of aquaculture producers was administered to elicit information about grower production techniques, grower opinions about the industry, and grower preferences for genetically improved fish stocks. Data from the survey were used to estimate a conditional mixed logit model to analyze grow-out producer preferences for selected genetic attributes. The attributes analyzed in the study were growth rate, disease resistance, and resistance to 10% lower dissolved oxygen levels. A price premium attribute was also included in the available alternatives. A conditional logit with interactions model was estimated and willingness-to-pay estimates were derived from the results. Improved growth rate was the most significant genetic attribute desired by grow-out producers. Willingness-to-pay estimates indicated that grow-out producers would pay 22% more relative to the current price of fingerlings to acquire a fish stock with a 20% increase in growth. Disease resistance and tolerance of low oxygen levels were not significant in the model. This implies that grow-out producers in the United States are more likely to adopt fish stocks with improved growth rates rather than those with disease resistance, or tolerance for low oxygen levels. The results also showed that the channel catfish and tilapia industries were more likely to adopt genetically improved fish stocks relative to other species. Future research that focuses on improved growth rates for these species are more likely to be adopted by United States aquaculture industries.

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Appendix: An Example of the Choice Task from Survey Version #3

Options "A" and "B" represent hypothetical fingerling stocks which are made up of the specific genetic characteristics listed below them. Please check the letter that indicates your **preferred option in each set**. If neither option is preferable, or if you prefer your current fish stock to either options "A" or "B," then select the "Neither" option under the table.

Choice Set 1

Attribute	Option A	Option B
Growth rate	20% better	Current
Disease resistance	Current	20% increase
Resistance to 10% lower dissolved oxygen levels	Current	Yes
Price premium	40%	40%

Please indicate the option that you would select if these products were made available to you in the marketplace. (Select one)

Option A ☐ **Option B** ☐ **Neither** ☐

Choice Set 2

Attribute	Option A	Option B
Growth rate	Current	20% better
Disease resistance	10% increase	20% increase
Resistance to 10% lower dissolved oxygen levels	Yes	Current
Price premium	20%	60%

Please indicate the option that you would select if these products were made available to you in the marketplace. (Select one)

Option A ☐ **Option B** ☐ **Neither** ☐

Risk Considerations in the Application of Cryopreservation Techniques for Aquatic Species

Vincent A. Mudrak and Gregory L. Looney

This chapter deals with the use of cryogenics for gamete preservation and potential risk to highly valued aquatic resources. The opinions expressed are not official policy of the U.S. Fish and Wildlife Service (USFWS), but reflect some of the concerns and ideas expressed by the Directors of the USFWS Fish Technology Centers, and other members of the USFWS who have taken an interest in the use of applied cryogenic technologies in fisheries work.

Introduction

The Southeast Region of the USFWS proposes to develop and refine technologies necessary to preserve important genetic resources needed for current and future fisheries programs. Accordingly, the Warm Springs Regional Fisheries Center, Warm Springs, Georgia, has initiated actions necessary to address the Region's fisheries program needs, and to establish a model repository for a nationwide cryopreservation program for fish gametes. These actions have been undertaken in a slow, deliberate fashion, making sure that the concerns of fisheries managers are taken into account. Plans now include an assessment of federal, state, and private fisheries needs for germplasm preservation. Subsequent implementation by the Southeast Region will proceed incrementally, in response to these needs.

Cryopreservation of gametes can be an important technical approach for conserving genetic resources and can provide opportunities for fisheries program expansion and development. The Southeast Region is taking care in assessing the risks of using specific cryogenic technologies, and ensuring that actions are responsible and prudent. Accordingly, applied cryogenic technologies will be developed to be compatible with good fisheries stewardship plans. Moreover, prospective benefits of cryopreservation technology will be reviewed in relation to enhancing conservation biology, strengthening the recovery and restoration of imperiled species, and improving capabilities to provide fish that meet research and recreational fisheries needs.

Applications and Benefits

The full range of benefits of cryogenic work cannot be realized until a program is implemented and evaluated. However, many fisheries managers and scientists are presently examining areas of applied cryogenic technology (Table 1). While the applications and benefits that can be derived from this new technology are endless, there is also another aspect that has an equally endless set of concerns. These are the risks that accompany the implementation of new technologies, as well as the risks associated with human error and poor human judgment. This brings us to choices and value systems, and the question of "how much risk are we willing to accept?". Risk is associated with every human activity. Risk can be real or perceived to be real. It is something that people view very differently -- and there is often no right or wrong viewpoint.

Mudrak, V. A. and G. L. Looney. 2011. Risk Considerations in the Application of Cryopreservation Techniques for Aquatic Species. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 931-938.

Table 1. Contemporary cryogenic applications and benefits for fisheries.

Applications	Benefits
Ensure the synchrony of fertile male and female gametes for spawning	Successful spawning even if sexes mature at different times
Employ germplasm when resource issues preclude broodstock collection in the field	Successful management of interjurisdictional fisheries
Access desirable traits of unique and important captively propagated fishes	Avoid inbreeding of broodstock and retain desirable traits
Accomplish population enhancement with fish species of very low abundance	Maximize effective population size of wild broodstock
Preserve genetic material of highly imperiled species as a recovery back-up	Maintain species integrity while habitat improves
Provide a source of genetic material that will yield similar offspring	Use for research tests (the “white rat” of fishes)
Develop a fish gene bank for specific pathogen-free germplasm	Use to avoid vertical transmission of pathogens
Increase quality control assurance for fish hatchery product evaluation	Propagate fish matched to program requirements
Maintain and use germplasm from distinct population segments	Comply with requirements of species recovery plans
Use fish germplasm from evolutionarily significant units	Produce fish adapted to specific habitats

Perceptions of Risk

Differences in risk perception probably relate to how we measure it. If we look at a risk issue and measure it in terms of consequences, the risk tends to be large. If we look at risk in terms of probabilities, the risk tends to be much smaller. For example, exposure of human skin to ultraviolet light in a laboratory might be viewed in terms of consequence, as a factor contributing to undesired health affects to the exposed person. The ***consequence*** is a potential undesired health affect, and some people would see high risk. However, looking at this same issue in terms of ***probability***, others might compare the risk of this exposure to common every d occurrences -- like an afternoon walk in the sunshine. Under this scenario, some people would assess the risk to be negligible.

In the world of fish culture, fisheries and aquatic resource management, we deal with people who possess philosophical value differences. These differences shift perceptions, and the

way people deal with aquatic resource issues and the risks associated with them. These are real value differences, and often reflect the paradigms that guide our personal perception of reality.

Paradigms are rules and boundaries to our thinking that predispose us to how we make decisions. Human predisposition and perception can cloud reality. For example, if we could journey back in time, we would see philosophers straining to explain the earth and natural forces. For centuries they held to a misleading paradigm, and in a geologic time-scale, it was only yesterday that we correctly determined the shape of the earth to be round and not flat.

If we move forward in time from the Middle Ages to post World War II, and ran a survey on the value of nuclear energy, we would see a wide range of opinions. Public opinion would probably be distributed along a bell-shaped curve, reflecting different degrees of perceived benefit and risk. On either end of the curve would be strongly opinionated people, willing to make their feelings known. In this case we could refer to them as the hawks and doves.

Hawks would view nuclear energy as something highly beneficial, a fabulous source of energy with tremendous opportunities and passport to the future (the sky is the limit). A Dove's view would be something totally different. They would envision an insidious "darker" side to this new energy source, with inevitable holocaust, radiation effects, widespread disease and a strong fear of approaching doomsday.

We can apply the previous analogy to the present d and examine cryogenics, and in particular, the cryopreservation of fish sperm. Society today is more complex than ever. New and advanced technologies are developing at an exponential rate. Things that we can now achieve are pushing us to new horizons. But along with this new wave of opportunity comes trial and error, and many groups of visionary people with mixed perceptions. Similar to the issue of nuclear energy, cryogenics is being viewed very differently by many people, and there seems to be some philosophical differences that tend to shift perceptions. Although no group should be stereotyped as being totally polarized in their opinions, people with a strong fish production orientation are apt to be very supportive of cryogenics work. These people tend to be technocrats eager to gain access to new technology, and eager to apply these technologies for human benefit. Contrastingly, people with a strong preservationist orientation will tend to see cryogenics work as having some high risk potential. Preservationists look at issues in terms of much longer time frames and seek to ensure that new technologies and quick-fix human applications will not impart irreversible damage to the living planet. Accordingly, while the production-oriented technocrat would probably view cryogenics as a wonderful opportunity, the preservationist view might be contrastingly different -- much more cautious, and possibly foreboding.

Technocrat View

-- Increased efficiency in meeting fishery program goals and human needs through improved husbandry and productivity (fish size, growth rate, disease resistance).
--Protection of germplasm of unique and highly valued fish stocks, and increased opportunity to select and use new genotypes and phenotypes.
--Increased availability of genetically diverse germplasm while reducing numbers of live brood fish held in captivity.

Preservationist View

--Potential loss of valuable genetic material during storage and through unproven techniques.
--Inevitable acceleration of the proliferation of unwanted hybrids influencing the genetics of native stocks.

--Probable pathogen transmission (vertical and horizontal) into regions historically free from the disease.

If we generated a bell-shaped curve for the perceptions of our fisheries constituency, we should see gradients of positions. Some people will view cryogenics as "Blue Sky and Nothing to be Concerned About." Some people will look at cryogenics work with caution, and "Bright Red Lights Will Flash in Their Minds." However, the vast majority of people will fall somewhere in between these extremes. These people will recognize potential risks associated with cryogenics work. They will also examine ways to negate these risks, or minimize them to acceptably low levels that the vast majority of people can accept.

The USFWS maintains a core of seven Fish Technology Centers. The authors polled the Fish Technology Centers, asking the staffs to think about cryopreservation of fish gametes, and to subsequently identify the kinds of risks that they, and others in their Regional area perceive to be associated with cryogenics. The following (Table 2) characterizes these risks into two categories: technology-based risks, and application-based risks.

Table 2. Perceived risks associated with cryogenic applications in fisheries.

Technology-based risks	
Labels	Loss of marks over time enhances chances for application error
Equipment	Malfunctions might corrupt the integrity of samples
Inventory	Computer glitches and improper data can add to errors in use
Storage	Loss of sperm viability may intensify genetic selection
Safety	Freeze "burn" and nitrogen gas narcosis are human concerns
Disease	Vertical and horizontal transfer of fish pathogens may increase
Genetics	Comet assay evidence that freeze-thawing sperm might alter nuclei DNA, (Labbe 2001).
Facilities	Space limiting factors can contribute to human error
Application-based risks	
Genetics	Propensity to rely on frozen sperm for several generations
Disease	Inadvertent selection of inferior, disease-susceptible fish
Hybrids	New "species" impacting natural populations and habitats
Habitat	"Trade-offs" as indirect loss of critical or essential habitats
Ethics	Disposal dilemma for "old" germplasm (T & E species)

Developing a Cryogenics Program to Minimize Risks

Potential management and risk scenarios should be examined closely before pursuing a cryogenics program. For the Southeast Region, we have recognized several questions that should be considered in order to minimize risks, when applying cryogenic principles to enhance fisheries and aquatic resource programs.

How Many Species Do We Need to Plan for, and are We Addressing the Right Species?

We are in the process of identifying high priority species for the Service, our state partners and non-government organizations that may be interested in retaining germplasm. Plans

must ensure that each species has enough storage space for multiple sets of frozen samples. Accordingly, active working germplasm repositories of a large size may be needed.

How Many Samples Do We Need to Store for Each Species?

To ensure genetic integrity some programs will require many sets of germplasm samples for a given species. Moreover, various sample volumes and dilutions will be required for specific research needs.

How do We Deal with Working and Archive Repositories?

We normally think and plan in lengths of time that correspond with annual cycles, seasonal use, or project duration of a few yr. These are short-term storage needs that require active working repositories. We must also be prepared to hold samples for much longer times. Longer term working repository storage is especially important for population recovery efforts that might take 20 or more yr. Moreover, for archived samples, we must think about saving genetic material for many yr, possibly as long as a century. This work is analogous to storing important genetic material in a time capsule.

How Do We Manage for Pathogen Control?

A separate repository is needed to maintain the integrity of samples determined to conform to a specific pathogen-free status. For many samples, we may need to screen and isolate germplasm in ways that will reduce the likelihood of transmission of an infectious agent. We do not want the gametes, or liquid nitrogen media (Clark, G. N. 1999, and Tabrizi et al. 1997), to serve as a disease vector. In certain situations, to minimize risk of inadvertent viral et al cross contamination, we might prefer storage in nitrogen vapor rather than liquid nitrogen.

How Do We Develop an Effective Inventory System?

All of us have probably had to deal with trying to find a specific nut or bolt from a mixed bag. If you recall the difficulty and time it takes to sort through the hardware, you will begin to appreciate the value of an inventory system. Accordingly, to proceed in serious professional fashion, it is important to develop a user-friendly germplasm repository and data base. Additionally, it is important to characterize traits and document the information for future use (Kincaid 2000). We believe it is necessary to ensure that we can identify and retrieve the correct samples whether they are needed next week, next yr, or next century. A sample identifier, plus an inventory and tracking system is a must.

What Do We Do with Old Samples?

This is a difficult question and has ethical ramifications (Wachtel and Tiersch 2000). From our perspective, most gametes in working germplasm repositories should be treated as important, but expendable samples (for propagation, research, etc.). However, certain samples may represent important material from special populations, or genetic material that may hold a valuable trait, and then this genetic material should also be entrusted to long-term archived storage. Also, once samples are brought into the archive system, they should only be discarded through established policy guidelines. Because of the commitment that must be made, sample entry must undergo some review process.

Many of these issues have previously been addressed by conservation workers in the recovery and management of mammals, plants and birds. These programs have established

guidelines that address conservation genetics and the management of cryogenic germplasm repositories. These guidelines are being continuously refined to resolve or answer new questions, including questions of risk. The standards that guide fishery workers should be equivalent to the same standards that guide other cryogenics work in conservation biology. We can learn much from what others have already experienced.

How Do We Ensure Human Safety?

The U.S. Fish and Wildlife Service employs safety standards and protocols that match industry guidelines for liquid nitrogen use and safety (Airgas MSDS 2007, and CSB 2003). Protocols for safe handling of liquid nitrogen should be developed and enforced as standard operating procedures, and adequate ventilation in confined space should ensure that oxygen levels remain appropriate.

Don't Blame the Tool!

We must recognize that cryogenics is a tool. When a tool is used wisely it can accomplish much good. Used unwisely, the same tool can be detrimental. We have all heard the statement: "It's not the gun that's the problem, but the shooter that uses it." The shooter determines the resultant effects. These effects can be good or bad. And so it is with cryogenics, we must learn to use the tool wisely. We must ensure that we retain and use our valuable genetic resources in a responsible fashion. Moreover, there are certain cryogenic issues that must be examined closely. In sensitive resource areas, such as working with imperiled species, people must communicate and plan their activities.

Undoubtedly science and technology will continue to advance, with concurrent increases in human capabilities to preserve and use gametes and embryos of many aquatic life forms. Along with these technological gains comes increased responsibility to manage genetic resources and to determine which genetic material will be maintained in perpetuity. Obviously as scientists and managers we have choices. We need to ensure that the approaches we take minimize risk to valuable aquatic resources. As cryogenic opportunities emerge let us be cognizant of the areas of high risk, as well as the areas of safe study. Accordingly, as we move into applied cryogenics, let us proceed on a course that takes us on a safe and responsible journey. There is much work to be done.

The USFWS is a lead federal agency for stewardship of the fishes, fisheries, aquatic wildlife and wildlife of the United States and is also charged with the preservation and recovery of endangered and threatened species and species of special concern. The USFWS protects wildlife for the public and enforces laws and regulations concerning fish and wildlife, including transportation across state lines, import of live exotic species, and verification of sterile exotic species. Under these authorities, the USFWS remains engaged in developing and implementing recovery and restoration plans for many imperiled species. However, as populations decline, valuable genetic resources can be lost.

Cryopreservation of gametes can be a valuable tool for improving the versatility and effectiveness of fisheries management. This is especially important for preservation of declining aquatic biological resources, by using stored genetic resources that can be applied immediately for recovery and management of imperiled, threatened, or endangered fishes. Techniques for freezing fish sperm are improving rapidly and cryopreserved sperm can be used in the production of genetically appropriate hatchery fish for restocking wild populations. Although the basic principles are fairly well understood, the specific requirements for successful cryopreservation of

sperm must be developed on a species-by-species basis. Preliminary studies of sperm cryopreservation had been conducted by the USFWS with the Apache trout *Oncorhynchus* Apache (David et al. 2000), razorback sucker *Xyrauchen texanus* (Tiersch et al. 1997, 1998, 2000) and the bonytail chub *Gila elegans* (Gorman 2000). And refinements in techniques continued, as work progressed with other species: pallid sturgeon *Scaphirhynchus albus* (Wayman et al 2008) and shortnose sturgeon *Acipenser brevirostrum* (Horvath et al. 2005). No attempts have been made to cryopreserve eggs or embryos of any of these species.

Techniques for collecting and freezing fish sperm and eggs in the field are only beginning to be explored. Adapting laboratory procedures for collection, evaluation, storage and freezing of fish sperm is critical to the successful cryopreservation and safeguarding of genetic resources. Therefore, analytical approaches must not only address the development of improved laboratory cryogenic techniques, but also address ways of resolving problems encountered during the application of these techniques in the field.

Applications of cryogenic technologies will undoubtedly be a challenge of the twenty-first century. The road that lies ahead will be demanding and rewarding. There is much to do. There is much to learn. Towards this end, we need to begin now to recognize our strengths, our weaknesses, our risks and our opportunities. Let us move forward with a resolve to develop strategies that encompass responsible aquatic resource applications. This is our challenge for the future. Let history record this work as a positive and lasting achievement.

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Infectious Disease and Quality Assurance Considerations for Transfer of Cryopreserved Fish Gametes

Jill A. Jenkins

Introduction

A well-designed program for the cryopreservation of sperm from aquatic species would incorporate a comprehensive strategy for collection and storage of high-quality gametes free of pathogenic contaminants. Unlike the artificial insemination industry for domestic livestock in which costly venereal diseases have been eliminated (Foote 1996), the rapidly expanding area of aquatic cryobiology must consider external fertilization. Because the presence of microorganisms in semen collected and stored for use in artificial breeding constitutes a potential hazard of disease transmission, minimizing these threats is a goal of a successful cryopreservation program.

Healthy breeding animals and broodstock are prerequisites for good gamete quality and for reduced microbial contamination. Although microorganisms are routinely present in semen of aquatic species, they can decrease semen quality (Saad et al. 1988, Jenkins and Tiersch 1997). *In vitro* tests of fresh and thawed samples can assist in judging sperm quality and in predicting fertilization success. Testing methods for specific pathogens may be warranted for discriminant testing of fish tissue and semen. Continual improvement in diagnostic techniques will allow greater accuracy in confirming the absence of microbes of concern, resulting in greater confidence in international transport of gametes.

Although cryopreservation of sperm has become an accepted technique for selective breeding and genetic improvement in livestock industries, no systematic approach is available for banking germplasm of aquatic species (i.e. embryos, semen and ova). The intent of this chapter is not to provide recommendations for specific measures to eliminate particular pathogens and subsequent diseases, but rather to develop a general framework and strategies for facing the new and unexpected. This chapter presents microbiological and quality assurance concerns for a cryopreservation program. In particular, the chapter identifies organisms transmittable in semen of animals, microorganisms and diseases of importance to aquatic species, pathogen detection issues, methods for prevention and control and how sperm quality can be assessed.

Disease Transfer with Sperm from Non-aquatic Species

Because disease agents are often isolated from semen of domestic livestock (Hare 1985) and aquatic species (Table 1), pathogen transmission via cryopreserved gametes is plausible. For this reason, national and international regulatory requirements and guidelines have been instituted (Jenkins, this volume) for the translocation of stored gametes. Several contaminants have been correlated with reduced fertility and have been found in fresh and processed semen (Thacker et al. 1984, Shin et al. 1986). For example, *Pseudomonas aeruginosa* has been associated with low fertility in bulls and can be transmitted in frozen milt (Getty and Ellis 1967).

Jenkins, J. A. 2011. Infectious Disease and Quality Assurance Considerations for the Transfer of Cryopreserved Fish Gametes. In: *Cryopreservation in Aquatic Species,* 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 939-959.

Table 1. Examples of microorganisms detected in semen.

Microbe	Host Animal	Reference
Mycoplasmas		
Mycoplasma meleagridis	turkey	Ferrier et al. 1982
M. bovis	bovine	Shin et al. 1986
M. bovigenitalium	bovine	Shin et al. 1986
Ureaplasma spp.	bovine	Shin et al. 1986
	turkey	Stipkovits et al. 1978
	bovine	Truscott and Huhnke 1984
Viruses		
pseudorabies virus	swine	Thacker et al. 1984
enteroviruses	swine	Thacker et al. 1984
foot-and-mouth disease virus	swine	Thacker et al. 1984
swine vesicular disease virus	swine	Thacker et al. 1984
African swine fever virus	swine	Thacker et al. 1984
pestis virus	swine	Herklots and Nasto 1998
human immunodeficiency virus	human	Martz et al. 1998
bovine viral diarrhea virus	bull	Revell et al 1988
infectious bovine rhinotracheitis	bull	Parsonson and Snowden 1975
Bacteria		
Escherichia coli	stallion, poultry	Jasko et al.1993, Sexton et al.1980
	swine	Thacker et al.1984
β-hemolytic *Streptococci* spp.	stallion, poultry	Jasko et al. 1993, Sexton et al.1980
	swine	Thacker et al.1984
Alcaligenes spp.	poultry	Sexton et al.1980
Bacillus spp.	poultry, swine	Sexton et al.1980,Thacker et al. 1984
Staphylococcus aureus	poultry, swine	Sexton et al.1980, Thacker et al. 1984
Proteus spp.	poultry, swine	Sexton et al. 1980, Thacker et al. 1984
Klebsiella pneumoniae	stallion	Jasko et al. 1993
Enterobacter spp.	swine	Thacker et al. 1984
Pseudomonas aeruginosa	stallion, bovine	Getty and Ellis 1967, Jasko et al 1993, Eaglesome and Garcia 1995
Pseudomonas spp.	channel catfish	Jenkins and Tiersch 1997
Aeromonas sp.	channel catfish	Jenkins and Tiersch 1997
Klebsiella sp.	channel catfish	Jenkins and Tiersch 1997
Pantoea sp.	channel catfish	Jenkins and Tiersch 1997
	swine	Thacker et al.1984
Campylobacter fetus	bull	Van Camp et al.1992
subsp. *venerealis*		Shin et al. 1986
Actinobacillus seminis	ram	Low et al. 1995
Haemophilus somnus	bovine	Shin et al. 1986
Leptospira spp.	swine, bull	Thacker et al.1984
Parasites		
Tritrichomonas fetus	bull	Van Camp et al. 1992
Chlamydia psittaci	buck, ram	Low et al. 1995
Rickettsia		Kruszewska and Tylewska-
Coxiella burnetti	bull, human	Wierzbanowska 1997

Microorganisms of Importance to Fish

Many microorganisms are essential for life on Earth and only those that multiply in or on a host and cause tissue damage are termed pathogens (Thune et al. 1993). Primary pathogens are those that induce infection in normal, healthy individuals. As with other species, fish that are exposed to infectious disease agents do not necessarily develop infections or the manifestation of clinical disease (LaPatra 1997) and environmental organisms typically produce disease only when host resistance is compromised. Studies to determine the impact of disease on free-ranging species have been limited, thus the potential impacts of disease in these populations have been inferred from the experience gained in intensive culture, which magnifies the effect of pathogens (Fryer and Bartholomew 1996, LaPatra 1997).

Approximately 50 species of bacteria and 30 viruses (Table 2) have been isolated from diseased fish (Fryer and Bartholomew 1996). Most bacteria are Gram negative, and most fish viruses belong to Reoviridae, although members of the Rhabdoviridae and Herpesviridae are the most important pathogens. Viral diseases are more infectious than bacterial or protozoal diseases and have a greater ability to surmount international barriers (Gibbs 1981). In fact, salmonid viral diseases have received the most attention in regulatory guidelines written about distribution of animals and gametes (U.S. Fish and Wildlife Service 1995, OIE Fish Diseases Commission 1995a, Code of Federal Regulations 1997).

The most common means for spreading disease is when infected fish come in contact with uninfected fish (Avault 1996) in horizontal transfer, but some disease agents can be transmitted vertically from parents to progeny. Some highly infectious pathogens are transmitted horizontally and vertically and do not respond to available antimicrobial agents (Fryer and Bartholomew 1996). Chronically infected carrier fish are difficult to detect and remove from healthy populations. The vertically transmitted disease microorganisms, some of which are highlighted below, elicit the most concern with regard to gamete storage and artificial breeding.

Bacterial Pathogens

Cytophaga psychrophila

Ensuring the quality of fertilized eggs is important for maximizing production of progeny. Certain bacteria have been associated with a reduction of fertilized eggs reaching the eyed stage. *Cytophaga psychrophila* has been isolated from batches of Atlantic salmon *Salmo salar* eggs that displayed poor survival (Cipriano et al. 1995), further implicating this bacterium in vertical transmission via eggs and sperm (Symula et al. 1990).

Renibacterium salmoninarum

The causative agent of bacterial kidney disease (BKD), this Gram positive bacterium has been disseminated by introductions and has been detected in cultured and feral salmonids with no history of human intervention (Fryer and Bartholomew 1996, Pascho et al. 1998). It is vertically transmitted via eggs from infected parents, which is unique for bacterial fish pathogens (Pascho et al. 1998). Treatment with antibiotics is

Table 2. Important fish diseases, hosts and ranges

Disease	Species affected	Range
Viral		
Viral hemorrhagic septicemia (VHSV)	salmonids, herring, cod	Northwest U.S., Europe
Infectious hematopoietic necrosis (IHNV)	salmonids	Northwest U.S., Europe, Japan
Infectious pancreatic necrosis (IPNV)	many species	
Landlocked salmon virus	masu salmon	
Japanese salmonid herpesvirus	salmonids	
Largemouth bass virus		Santee-Cooper Reservoir, South Carolina
White sturgeon adenovirus disease	white sturgeon	
Spring viremia of carp (*Rhabdovirus carpio*)	common carp	Europe, Middle East, Southeast U.S.
Channel catfish virus	ictalurid catfish	North America
Walleye diffuse epidermal hyperplasia		worldwide
Lymphocystis	freshwater and marine fish	China, East and West Europe
Pike fry rhabdovirus disease	northern pike	Japan
White sturgeon iridovirus	centrarchids	walleye
Oncogenic viruses	freshwater and marine fish	Northwest U.S., California, Canada
		California
Protozoan		
Ceratomyxosis (*Ceratomyxa shasta*)	salmonids	
Proliferative kidney disease	salmonids	worldwide, Northwest U.S.
Whirling disease (*Myxobolus cerebralis*)	young salmonids	Northwest U.S., Europe
Microsporidosis (*Enterocytozoon salmonis*)	salmonids	worldwide
Proliferative kidney disease (myxosporidian)	salmonids	worldwide, worldwide
Proliferative gill disease	channel catfish	Southeast U.S.
Ich (*Ichthyophthirius multifilis*)	freshwater and marine fish	freshwater and estuaries
		sturgeon
Bacterial		
Bacterial kidney disease	salmonids	worldwide, North America and Europe
(*Renibacterium salmoninarum*)		
		worldwide

Table 2. (Continued)

Disease	Species affected	Range
Bacterial (continued)		
Furunculosis (*Aeromonas salmonicida*)	variety of species	worldwide, fresh, brackish
Enteric redmouth (*Yersinia ruckeri*)	salmonids and others	
Motile aeromonad septicemia (*Aeromonas hydrophila*)	goldfish, carp, shad	worldwide
Bacterial tail rot (*Pseudomonas fluorescens*)	many species	Central, Eastern U.S.; Japan (estuaries)
Epitheliocystis disease (*Chlamydia*)	freshwater and marine fish	Alabama, Florida (estuaries)
Pasteurellosis (*Pasteurella piscicida*)	striped bass, white perch	worldwide
Streptococcosis (*Streptococcus* spp.)	freshwater fish	
Vibriosis (*Vibrio anguillarum, V. ordalii*)	oysters and fish	
Columnaris (*Flavobacterium columnare*)	warm- and coldwater species	worldwide
Enteric septicemia of catfish (*Edwardsiella ictaluri*)	channel catfish	Southeast U.S.
Other		
Rickettsiosis (*Piscirickettsia salmonis*)	salmonids	worldwide
Saprolegniasis (*Saprolegnia* spp.)	freshwater fish	worldwide

ineffective and no vaccine is available. Vertical transmission can be interrupted by destroying fertilized eggs from brood fish most likely to transmit the disease. The pathogen is slow-growing, thus low-level infections may not be identified before hatchery populations are released, allowing infected salmonids into the wild.

Viral Pathogens

Infectious Hematopoetic Necrosis Virus (IHNV)

This is one of the most important viral diseases of salmonids, especially among juvenile fish where losses can be high (Winton 1991). In addition to fish-to-fish transmission and environmental contamination, IHNV can be transmitted with eggs, disinfected or not (USFWS 1995). The only control measures for IHNV are avoiding exposure to the virus, destroying infected stocks and using virus-free water supplies and certified eggs (Winton 1991).

Infectious Pancreatic Necrosis Virus (IPNV)

This virus threatens a variety of fish species. Once infected, the fish may become lifelong virus carriers. The U.S. Fish and Wildlife Service has allowed for the transfer of eggs from infected fish to USFWS facilities if all eggs are water-hardened in an iodine disinfectant and incubated in virus-free water throughout the period prior to transfer, provided the transfer would not threaten the species at the hatchery (USFWS 1995).

Viral Haemorrhagic Septicemia Virus (VHSV)

This is a cold-water rhabdovius agent of several saltwater species. Survivors of VHSV may become asymptomatic carriers. The carrier state frequently shifts to high shedding of virus at times of spawning. Virulent virus is shed in the feces, urine and sexual fluids (OIE 1997).

Oncorhynchus masou *Virus Disease (OMV)*

This is an untreatable exotic disease not detected in the United States, which infects salmonids in Japan and Eastern Asia. There is a septicemic phase of the infection, followed by a carrier state that leads to virus shedding via the sexual products at times of spawning (OIE 1997).

Rickettsia

Although intracellular rickettsial pathogens have been found in the gonads of infected fish, vertical transmission of the most characterized species, *Piscirickettsia salmonis*, has not been clearly demonstrated (Fryer and Mauel 1997). Rickettsial pathogens affect fish over broad host and geographic ranges in freshwater and marine environments (Fryer and Mauel 1997).

Microorganisms detected in (or suspected to be in) gamete samples of aquatic species could be ranked according to their disease transmission risk as defined by national and international guidelines. Organisms that are cause for notification and certification by Office International des Epizooties (OIE) would be candidates (Table 1) (Jenkins, this volume). Only then can informed decisions be made as to whether the gametes should be tested for pathogen presence before use and shipping. An example from the livestock industry is the opportunistic pathogen *Pseudomonas aeruginosa*, whose presence in processed bovine semen samples precludes use of the semen for artificial breeding regardless of whether the number of organisms in the inseminating dose could cause a pathogenic effect (Eaglesome and Garcia 1995). A sec

example is the restriction of gametes for export from cows and bulls infected with the bluetongue virus serotype 20 (Parsonson et al. 1987).

Pathogen Detection

For human sperm donor programs, infectious-disease testing has proven to be cumbersome and sometimes ineffective (Zilberstein et al. 1997, Martz et al. 1998). Indiscriminant disease screening of aquatic species to determine which gametes should undergo cryopreservation may not only be ineffective at detecting microbes (Thacker et al. 1984), but also would be unrealistic. Additionally, it may prove difficult to pinpoint the host or the environment as the origin of the contaminating microorganism in a gamete sample.

Until definitive studies are conducted on the source, reservoir and normal mode of transmission, microorganisms may be considered as possible contaminants of stored gametes from fish of the appropriate history and locale. The USFWS has been entrusted to ensure that gametes, fertilized eggs, and fish are shipped or accepted only in compliance with the Fish Health Policy (USFWS 1995), area disease control programs, regional stock transfer policies and state regulations. See Jenkins (this volume) for a list of microorganisms for which the USFWS performs tests. The choices for testing microorganisms can be made through following these policies as well as the OIE guidelines.

Methods employed for the detection of microorganisms should be well standardized, economical, sensitive and specific. For inspecting fish for microorganisms, the appropriate procedures are outlined in the current edition of the *American Fisheries Society Fish Health Blue Book: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens* (AFS Fish Health Section 1997). For virus detection, minimum procedures for federal facilities are outlined in the USFWS Fish Health Policy. *The Diagnostic Manual for Aquatic Animal Diseases* (OIE 1997b) provides directions for notifiable and significant diseases of fish, as well as diseases of bivalve molluscs and crustaceans. This manual also provides a list of reference laboratories for fish, mollusc and crustacean diseases and organizations that have a cooperative agreement with the OIE. Procedure modifications are needed for testing unprocessed and processed sperm samples. Experimental infection and consequent detection of antigen or antibodies in milt or ovarian fluid would yield definitive information for making decisions about gamete use and transfer (Parsonson et al. 1987). Results of the tests should be evaluated relative to the disease status of the hatchery, or exporting or importing countries, the history of the donor stock, and the known epidemiology and pathogenesis of the potential disease agent.

Detecting Microbes in Culture

Even if present in a sample, some disease agents elude detection because of their low numbers or the limits of detection and culture technology. Some bacteria have been found to be viable, but dormant or not recoverable on conventional bacteriological media routinely used to isolate and maintain cells in culture. One example with implications for aquatic ecosystems is *Vibrio cholerae*. When bacteria are in a viable, but non-culturable state, the cells do not die when discharged into aquatic environments but instead remain viable and capable of transforming into a culturable state if environmental conditions become favorable (Huq et al. 1990, Stretton et al. 1997). Under such circumstances it may be prudent to take preventative measures. The cattle industry adds antibiotics to cattle semen prior to freezing because diagnostic techniques for pathogenic leptospirae are inefficient (Philpott 1993).

The laboratory operations needed to identify and characterize microorganisms are the isolation of the agents in culture, biochemical testing, and the molecular and immunological technologies used to identify agents that cannot be easily cultivated. The polymerase chain reaction (PCR) enables detection of specific gene sequences from microorganisms that are present in samples at extremely low concentrations (e.g. *Leptospira* spp. in bovine semen) (Masri et al. 1997). Several nested PCR assays have been successful at identifying microbes that are difficult to culture (McIntosh et al. 1996, Fryer and Mauel 1997, Pascho et al. 1998).

If required, testing of the susceptibility of fish gametes or stocks to specific pathogens could be used to assign risk. This would allow for testing gametes or stocks for deleterious consequences prior to moving forward with cryopreservation efforts. Cohabitation experiments under laboratory conditions could measure the potential for horizontal and vertical transmission.

Detecting Microbes in Donor Animals

Subclinical disease is more difficult to characterize than clinical disease and can only be detected with the assistance of diagnostic tests. The presence or detection of any infectious agent does not imply the presence of disease, and freedom from clinical disease is not an indication of freedom from infection. The question remains -- when should an animal to be used for gamete collection be tested for microorganisms? Again, the tests should be conducted relative to the disease status of the hatchery, exporting and importing countries, the history of the donor stock, and the known epidemiology or pathogenesis of the potential disease agent. Specific guidelines are provided in the USFWS Fish Health Policy (USFWS 1995) and the OIE Code (OIE 1997a). The laboratory examination could be directed toward the detection of prior exposure to the microorganism specified or to the detection of persistent infection. Examination of the donor, in addition to the semen sample, is recommended for virus testing (Gibbs 1981).

Detecting Microbes in Semen

In some cases, it will be necessary to test semen for a specific contaminant before distribution, such as with the virus that causes foot-and-mouth disease in cattle (Philpott 1993). However, microbiological analysis of semen is difficult. Bacteria are numerous, resulting in contamination of cell culture systems for virus isolation, and overgrowth of special media for slow-growing fastidious organisms (Carroll and Hoerlein 1972). Interpretation of pathogenic versus nonpathogenic bacterial isolates is confusing, and even periodic sampling of semen for specific contaminants may not prove useful (Thacker et al. 1984). Because the isolation of viruses from mammalian semen is complicated by the presence of antiviral factors in the seminal plasma (Polakoski and Kopta 1982) that may inhibit the ability of cell cultures to support virus replication, animal inoculations may be a more sensitive technique than cell culture for detecting viruses (Thacker et al. 1984). Antimicrobial activity against several bacterial species has been noted in bovine seminal plasma (Schollum et al. 1977). A productive research direction would be the design of sensitive and specific genetic probes for diagnosis of specific pathogens in milt and ovarian fluid.

Detecting Microbes in Thawed Samples

Thawed samples may be useful for detecting disease agents. Bovine viral diarrhea virus was undetectable prior to freezing, but was recovered after storage in liquid nitrogen (LN_2), suggesting that fresh samples may not be suitable for the efficient detection of that particular virus (Revell et al. 1988).

Reference Specimens

Another valuable component of the pathogen detection process for a sperm-banking program for aquatic species is the accumulation and maintenance of reference specimens (e.g. serum, tissue, pathogen isolates, specific antibodies and gametes inoculated with varying amounts of microbes) for comparative analyses. Clearly, if molecular epidemiology studies are to be performed in the future, specimen banks will needed.

Prevention and Control

Preventing the spread of microbial agents via stored gametes from aquatic species depends on careful techniques, including collection and handling of gametes, screening of broodstock for pathogens or pathogen exposure (and possible broodstock segregation), and prudent production and distribution of young. Specific broodstock management practices are available for suspect diseased animals (USFWS 1995).

To increase the safety of seafood by decreasing contaminant numbers, a hazard analysis critical control point (HACCP) program is operating. This program is an alternative approach to quality control where particular points along the seafood production pathway are singled out as likely places for contamination to occur. Once these critical control points are identified, steps are taken to prevent anticipated problems. A similar approach might be adapted for a cryopreservation program for aquatic species gametes.

Microbial agents, pathogenic or not, can be introduced at any point from the collection of gametes to dissemination of young. Non-pathogens cryopreserved and introduced into a new environment in the future may be exposed to conditions engendering pathogenicity. Therefore, precautions for minimizing or eliminating introduction of microbes must be the standard.

Among the procedures that merit evaluation are universal microbiological aseptic precautions for sample handling, use of disinfectants and other microbial reduction steps. A Section of the OIE Code (OIE 1997a) is devoted to methods of egg disinfection for fish farms, mollusk farms, and crustacean farms. The USFWS Fish Health Policy provides specific guidelines for reducing risks of pathogen transfer with respect to the transfer or handling of gametes. Additionally, the necessary use of antimicrobial agents with stored gametes may be considered a valid mechanism for microbial inhibition.

Addition of Antibiotics

Multiplication of microorganisms may be suppressed, but not stopped by refrigeration, and microorganisms are not necessarily killed by freezing. To control infectious agents that are transmissible with sperm, the addition of antibiotics is common in breeding practices with cattle (Shin et al. 1986, 1988, Eaglesome and Garcia 1995), stallions (Jasko et al. 1993) and poultry (Sexton et al. 1980). Some standard combinations of antibiotics have been delineated for the control of microorganisms that are not detrimental to seminal quality or fertility (Sexton et al. 1980, Lorton et al. 1988a, 1988b). Conception rates were 15% higher in cows inseminated with semen that was treated with gentamicin after it was thawed, compared with cows inseminated with semen treated with penicillin and streptomycin or with untreated semen (Stoianov 1987). However, cryoprotectants may render antibiotics less effective (Bartlett 1991).

Inhibition of bacterial growth by addition of antibiotics to refrigerated fish sperm (Stoss 1983, Stoss and Refstie 1983, Saad et al. 1988) has had favorable effects on viability and storage

time (Christensen and Tiersch 1996). One of the few studies performed on the bacteriological evaluations of fish sperm clearly demonstrated that morphologic changes and reduced motility of channel catfish sperm were coincident with increased bacterial numbers (Jenkins and Tiersch 1997). The addition of antimicrobials to stored sperm will minimize bacterially produced toxins, which may damage the sperm (Lake 1978). The addition of antimycotics would be useful for preventing mold growth in diluents.

By defining microbial populations, antibiotics can be selected to test against target microbial species (Jenkins and Tiersch 1997). Research is required to identify the antibiotics and antibiotic combinations at concentrations that will control the microorganisms found in fish semen. Studies can be designed to experimentally contaminate sperm samples to test the effectiveness of adding antimicrobial drugs before freezing and after thawing, antibiotic concentrations, times for incubation, and influence of extender (Pfutzner 1989, Eaglesome and Garcia 1995). Once defined, efficacious combinations and concentrations of antimicrobials will remove concerns that microorganisms will decrease sperm quality or be translocated. However, antibiotic usage should not be indiscriminate, because the therapeutic use of antimicrobial agents creates a selective pressure favoring survival of resistant strains (Wilson 1994).

Antibiotic Administration

The bacterial flora of animal semen contains various non-pathogens, potential pathogens, and known pathogens. Attempts have been made to reduce infertility induced by pathogens through the systematic administration of antibiotics to domestic animals (Thacker et al. 1984) and by the injection of adjuvanted bacterins (killed bacterial cells in suspension with substances that enhance the immune response) (Carroll and Hoerlein 1972). These methodologies may prove useful for aquatic species.

Evaluating Sperm Quality

Although standardized methods have been proposed for the study of human sperm (World Health Organization 1992), no such procedures have been developed to standardize semen analysis of domestic species (Gravance and Davis 1995). Semen parameters vary among animals of different species and stocks, within stocks, and even in different samples from the same animal. Therefore, tests on seminal quality of fresh and thawed samples are used extensively for quality control in research on seminal physiology and in commercial artificial breeding organizations (Van Camp et al. 1992). Prediction of fertilizing potential using mammalian sperm is usually based on *in vitro* assessments, yet there is no single test applicable to fresh and frozen semen that can predict fertility (Linde-Forsberg and Forsberg 1989, Fiser et al. 1991).

Quality assessments are performed on fresh, refrigerated and thawed semen. Evaluation of stored avian sperm is critical because fertilizing capacity lessens within 24 to 48 hr (Wishart 1989). Because *in vitro* tests useful for fresh avian sperm are not useful with thawed sperm, an accurate assessment must be done with a time-consuming fertility trial (Wishart 1995). With regard to aquatic species, basic research is essential for the development of reliable protocols for quality assessment of semen.

Classical methods of assessment of semen quality for mammals include sperm motility, acrosomal status and cellular and head morphology. Because repeatable assessments can be achieved only with increased assay objectivity and numbers of cells analyzed (Graham et al.

1990, Cloud and Kersten 1996), flow cytometry is a useful tool that is being applied to a growing number of investigations using reproductive cells. Generally, assessment of semen requires evaluation by a combination of these different *in vitro* tests (Graham et al. 1990, Ericsson et al. 1990, 1993) and a variety of tests are described below.

Fertilization Rate

The ultimate measure of sperm quality is fertilizing ability, but fertility trials are costly and not timely or practical. Estimating the number of fertilized eggs that reach the eyed stage (Aas et al. 1991) is done after a period of d, whereas laboratory methods provide immediate quality assessments.

Morphology

Sperm morphology assessment is one laboratory test that bovine semen undergoes before use in artificial insemination (Sailer et al. 1996). However, a clear predictive relationship between abnormal morphology of sperm heads and fertility does not always exist (Sharlin et al. 1979, Van Camp et al. 1992, Ericsson et al. 1993). Chromosomal material is tightly compacted in the head with nucleoproteins, and an acrosome is located on the outside of sperm of non-teleost species. This bag-like organelle contains lytic enzymes required for sperm to penetrate the oocyte, thus acrosomal status has been demonstrated to be related to fertility (Ericsson et al. 1993, Peralta 1993). Because sperm of only a few aquatic species (e.g. sturgeon and oysters) have acrosomes, the test for acrosome reaction (Graham et al. 1990) is not useful for teleost fishes.

Motility

To be motile and able to fertilize, the spermatozoon must be structurally normal and viable. Motility, although not always correlated with fertilizing ability (Aas et al. 1991, Ericsson et al. 1993), provides good evidence of functional integrity. Motility determinations made by microscopy may be variable due to the subjectivity of the evaluations and the short duration of motility of fish sperm (McNiven et al. 1992). Computer-assisted motion analysis techniques have been adapted for use with fish sperm (Toth et al. 1995). The structure responsible for motility, the tail, is fueled by mitochondria. Damage to mitochondria could be responsible for a decrease in the percentage of motile sperm (Morisson et al. 1997). The proportion of bovine sperm with functional mitochondria has been correlated with the percentage of sperm with normal morphology and motility, but not with estimates of fertility (Ericsson et al. 1993).

Sample Volume

Sperm concentration, or spermatocrits (number of cells/unit volume), is of concern in the quality control of samples because it provides assurance that the straws of extended semen contain the sperm numbers indicated (Evenson et al. 1993). Generally, higher numbers of cyropreserved sperm are needed to attain fertility rates similar to those of fresh semen. Although turbidometric and electronic counting methods have been used, flow cytometric techniques offer advantages, including distinct resolution between sperm and other particles such as bacteria (Evenson et al. 1993). Semen concentration has not been positively correlated with fertility in the bull, but spermatocrits are performed routinely for rams and bucks (Van Camp et al. 1992). Sperm concentration of turbot *Scophthalmus maximus* was difficult to estimate by spermatocrit, but spectrophotometry was found to be useful (Suquet et al. 1992). Fluorometric techniques have been used to measure concentration and viability of avian sperm (Bilgili and Renden 1974).

Viability

The sperm plasma membrane is one of the main structures affected by cryopreservation (de Baulny et al. 1997) and is an important component in the maintenance of sperm viability (Cloud and Kersten 1996). Differential supravital staining procedures, which depend on intact membranes to exclude stain, have been used to differentiate live from dead cells (Graham et al. 1990, Donoghue et al. 1995). Because cryoprotectants can interfere with supravital stains (Garner et al. 1986), other methods may better assess the viability of thawed samples. Simultaneous staining with two or more fluorescent stains (such as fluorescein diacetate derivatives, rhodamine 123 and reduced nucleic acid stains) targets not only live cells and those with compromised membrane integrity, but also additional aspects of cellular function such as mitochondrial function (Graham et al. 1990, Ericsson et al. 1993, Garner et al. 1994).

Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA), which uses the metachromatic properties of acridine orange to monitor the susceptibility of sperm chromatin DNA to acid-induced denaturation *in situ* (Evenson and Jost 1994), is a valuable test for fertility and reproductive toxicology studies (Evenson et al. 1994, 1995, Sailer et al. 1996). Computerized digital image analysis was recently used to compare sperm head morphology with the SCSA and fertility rankings of bull semen samples; variations within a sample were determined to be significantly related to sperm chromatin structure and fertility potential (Sailer et al. 1996). The SCSA has proven to be an objective and technically sound measure of semen quality with avian and mammalian sperm. It is used to evaluate the fertilizing potential of thawed bovine semen (Evenson et al. 1994), and it could prove useful with sperm from aquatic species.

Stress Tests

The "thermal stress test" has been used to estimate the capacity of sperm to survive in the reproductive tract of female swine and retain fertilizing potential (Fiser et al. 1991). This assay involves *in vitro* incubation for several hr followed by re-examination of semen and is useful in revealing damage that is not apparent immediately after sperm collection and processing. A sec stress test evaluates hypo-osmotic swelling that allows for the detection of viable spermatozoa with intact membranes (Jeyendran et al. 1984, Donoghue et al.1996).

Seminal Plasma

Seminal plasma is a nutritional transport medium for sperm consisting of cells, cellular particles, electrolytes, amino acids and proteins, with a particular pH, volume, sperm density and osmolarity/species (Peralta 1993). Human and mammalian semen contains a number of constituents with bacteriostatic properties, such as zinc, spermine, lysozyme, glucosidases and secretory immunoglobulin (Polakoski and Kopta 1982, Thacker et al. 1984). Bovine seminal plasma was found to have considerable antimicrobial activity against several Gram positive and Gram negative bacterial species, and against some mycoplasma.

Many investigators believe that seminal plasma can influence the fertilizing potential of spermatozoa. The influence of the acidic seminal fluid protein has been examined on bovine sperm viability and motility (Schoneck et al. 1996). It has been used to assess sperm viability as indicated by cytoplasmic enzyme leakage (such as dehydrogenase) from damaged sperm of rainbow trout *Oncorhynchus mykiss* (McNiven et al. 1992), and ion and sugar concentrations

have been evaluated in relation to fertilization rate in salmon species (Aas et al. 1991). However, few reports (Thurston et al. 1992) indicate seminal plasma to be useful in distinguishing between acceptable and unusable semen samples.

Because the determination of sperm function and its relationship to seminal quality and fertility is the goal by which all quality assurance tests must be measured (Garner et al. 1994), research is needed develop and optimize predictive *in vitro* tests for sperm from particular species. See Figure 1 for a schematic flow chart denoting when to test for microbes and sperm quality in the procurement and transportation of gametes.

Conclusions

Transport of fresh and frozen gametes from aquatic species dictates consideration of potential disease transmission. At the outset, a program for banking gametes from aquatic species must incorporate an appropriately formulated and internationally accepted scheme for the collection, storage and transfer of high-quality gametes free of microbial threats. The new cryopreservation discipline applied to aquatic species can take lessons from the domestic livestock industries and from operative fish health management policies. Although the risk may be minimal, the impact of semen contaminated with known vertically transmitted pathogens, or with potential pathogens, could be extreme.

Following quality assurance procedures particular to the gamete type and risk potential will limit the degradation of gamete quality and enhance the assurance that samples exclude specific pathogens and contain only minimal numbers of other microorganisms. Maintaining high integrity in a cryopreservation program will ensure animal health is not compromised and expand the use of cryopreserved gametes. Optimized cryopreservation techniques with subsequent fertilization free from disease will assist in the collaborative efforts of laboratory scientists, field scientists, resource managers and ecologists in securing the future of healthy genetic resources.

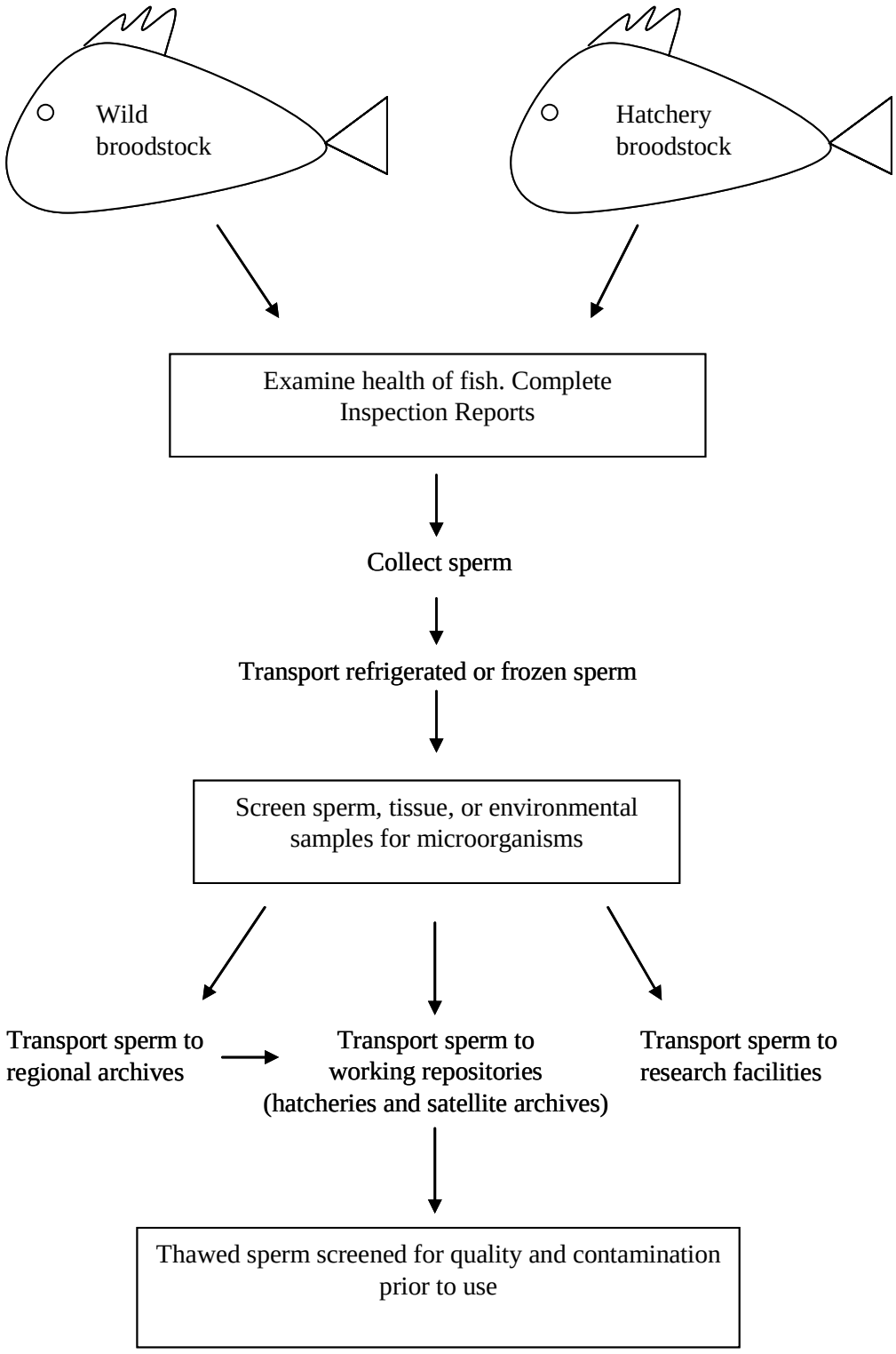

Figure 1. Steps in the procurement and transportation of aquatic species gametes intended for refrigerated or cryopreserved storage. Boxes denote points at which diagnostic testing for microbes of concern would yield benefits.

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Annotated Bibliography of Developments in the Last Decade

Few articles have been published on the subject of disease transfer or quality assurance in aquatic species sperm. However, the broader concept of biosecurity has become prominent since 2000, especially with increased international transportation of agricultural products. A keyword search resulted in 127 articles and 5 books discussing biosecurity since 2000, with only 14 of those articles and 2 books directed to aquatic sciences. A selection of articles is added below on disease transfer in sperm and biosecurity. Clearly, additional work is necessary to incorporate the concepts of biosecurity into the field of cryopreservation in aquatic species.

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Tiersch, T. T. and J. A. Jenkins. 2003. Biosecurity and regulatory considerations for transfer of cryopreserved sperm and early life stages of aquatic species. *In*: Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. C. -S. Lee and P. J. O'Bryen, editors. The World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. 171-198.

Regulatory Considerations for Global Transfer of Cryopreserved Fish Gametes

Jill A. Jenkins

Introduction

Federal and state resource managers, scientists, lawmakers, business and development investors, and the general public all struggle with issues surrounding the conservation of our biological heritage, especially in the face of increased population growth and consequent anthropogenic disturbances. Conservation interests include recovering exploited aquatic populations, decreasing the loss of genetic diversity, and reintroducing locally depleted species. However, research on husbandry and other techniques critical to implementing conservation strategies is often not started until few individuals remain. A program in the cryopreservation of gametes and embryos from aquatic species would address several of these conservation concerns by allowing the establishment of gene banks.

The potential problems that arise from the introduction or re-establishment of aquatic species illustrate the importance of taking a proactive approach against geographic translocations of potential disease agents. A small input of pathogens into fragile ecosystems could lead to large changes, and conservation programs are increasingly realizing potential risks involved with translocating pathogens along with host species (Cunningham 1996). The presence of microorganisms in archival samples of gametes from aquatic species could jeopardize valuable germplasm resources by lowering cell quality, as well as providing the potential for the dissemination of disease agents to and from wild or hatchery sources.

In the world economy, the international trade in animals and animal products necessitates that veterinary regulations be designed to prevent the spread of transmissible diseases to animals and humans, and among groups of animals. The guidelines yet to be formulated for international trade of cryopreserved germplasm of aquatic species should be easily integrated into the working policies of functional regional, national and international organizations involved with animal and animal product trade. Program development should take advantage of the historical delineation of health management responsibilities of these programs, while taking into consideration that a coherent, integrated program is currently lacking for fish health management policies in the United States. The objectives of this chapter are: 1) to discuss many of the available approaches, policies and plans with infrastructures that can be used as models in the establishment of a program in the long-term storage and use of germplasm from aquatic species; 2) to discuss regulations and practices currently in place for use of gametes from livestock and for minimizing the transfer of disease agents with salmonids and fish products (a look at such ventures in cryopreservation and gamete transfers provides a context for this chapter); 3) to address the importance of data management in expediting an efficient program in the collection and storage of gametes free from microbial threats, and 4) to address the concept of emerging diseases in wildlife and fish.

Jenkins, J. A. 2011. Regulatory Considerations for Global Transfer of Cryopreserved Fish Gametes. .In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 960-976.

Historical Considerations

Aquatic Species

Cryopreservation of fish spermatozoa has closely followed the technological developments in cryopreservation of mammalian gametes (Rana 1995). Soon after the cryoprotective value of glycerol was discovered (Polge et al. 1949), sperm of Atlantic herring *Clupea harengus* was cryopreserved (Blaxter 1953). Since the 1970's, cryopreservation literature has advocated a number of protocols for over 50 fish species, with most publications focused on salmonids, tilapia and carp (Rana 1995).

Avian Species

The initial work in cryopreservation technology began with the production of live chicks from domestic fowl hens inseminated with spermatozoa that had been frozen (Polge 1951). Since then, most studies have used the domestic chicken, but there are several reports on the successful cryopreservation of sperm from a number of nondomesticated birds (see Gee and Temple 1978, Gee 1983, Wishart 1995). Ducks and cranes have also been used due to the increased emphasis on captive propagation and gene pool preservation of birds threatened with extinction. Because of the agricultural importance of poultry, sperm from chicken and turkey have received the most attention regarding standardization procedures. Collected semen from birds has been used to detect disease agents (Thurston et al. 1975, Stipkovits et al. 1978, Ferrier et al. 1982). To decrease the spread of diseases to females and to diminish the production of bacterial toxins, antibacterial agents are sometimes incorporated into diluents for avian semen (Lake 1978). Long-term storage techniques with bird milt has allowed the safe transfer of milt without exposing females to known diseases (Lake 1978).

Livestock

Artificial insemination with livestock began in the late 1940's and is the most notable example of a successful combination of research, development and widespread application (Foote 1996). Consequently, this industry can provide clear examples of problems faced and approaches taken that are applicable to a cryopreservation program with gametes of aquatic animals.

The international transport of special breeds of domesticated animals (cattle, sheep, pigs and horses) and germplasm (embryos, semen and ova) has increased dramatically over the past 20 yr (Gibbs 1981). Artificial insemination was originally undertaken to eliminate venereal disease and to distribute advantageous genes. However, pathogen transfer can occur by the venereal route or by the use of semen or embryos. Semen used for artificial insemination has a great potential for spreading infectious diseases (Colenbrander et al. 1993). Reviews on disease transmission by semen and embryo transfer technology include those by Wrathall (1987), Afshar and Eaglesome (1990) and Philpott (1993).

To minimize the risk of pathogen dispersal, the international movement of livestock and germplasm is usually controlled through various forms of legislation and testing (Gibbs 1981, Colenbrander et al. 1993). The Office International des Epizooties (OIE), created in 1924 and headquartered in Paris, France, has been a leader in defining international health standards for terrestrial animals. Governments are informed of the occurrence and course of animal diseases, as well as ways to control them. Coordinated studies are also devoted to their surveillance and control. The OIE developed guidelines to work toward disease-free mammalian donors with semen free from potential pathogens. The International Animal Health Code Commission, one of

the OIE specialist commissions, deals with regulatory issues. Reference laboratories and collaborating centers provide OIE member countries with scientific and technical advice concerning disease surveillance and control. In addition to establishing international standards, the OIE has addressed issues such as diseases transmissible by semen and embryo transfer techniques (Hare 1985) and rules for trade in animals and animal products (OIE 1986).

Since the 1980's, the European Union (EU) has encouraged cooperation between countries and a level of international health standardization has been achieved (Gibbs 1981). A regulation requiring the addition of antibiotics to sperm meant for artificial insemination is legally prescribed within the EU (Colenbrander et al. 1993). The United States follows standardization under the National Association of Animal Breeders (NAAB) in Columbia, Missouri (NAAB 1995). Certified Semen Services, Inc. (CSS), a subsidiary of the NAAB trade association, has established minimum requirements for disease control of semen produced for artificial insemination. These requirements provide a minimum industry standard for health management. Due to the operations of CSS, the artificial animal breeding industry regulates itself without direct government involvement.

Programs to Consult

Just as disease risks should be assessed and precautions taken to minimize risks before wildlife is translocated (Cunningham 1996), similar efforts should be made with cryopreserved gametes intended for aquatic species production. Each aspect of the cryopreservation program should be reviewed by member agencies, including federal, provincial, and state government agencies, or other interested parties participating in cyopreservation activities (e.g. research). Member agencies can then establish and integrate appropriate policies into their operating approaches.

Fish Diseases Commission of the OIE

The OIE, the world organization for animal health (http://www.oie.int), harmonizes regulations for trade in animals, including animal and aquaculture products, for nearly 150 member countries. Member delegates are the country’s chief veterinary officer. The OIE has considered disease matters in aquatic animals since 1960 through its Fish Diseases Commission (FDC), which compiles information on diseases of fish, crustaceans and mollusks and on methods of disease control. The FDC has drafted a *Diagnostic Manual for Aquatic Animal Diseases* (OIE Fish Diseases Commission 1997a) and *International Aquatic Animal Health Code* (OIE Fish Diseases Commission 1997b). The FDC code contains information on international trade in fish, mollusks and crustaceans, including sections on import risk analysis and import/export procedures. Models of international certificates for trade in live and dead aquatic animals and gametes are also provided in the FDC code. Diseases that qualify for notification to the OIE and other significant diseases are presented in Table 1. The FDC manual provides recommendations for diagnostic methods for all diseases that appear in the FDC code.

Table 1. Aquatic animal diseases and their causative agents that are notifiable to the Office International des Epizooties[a].

Disease	Causative agents
Significant diseases of fish	
Epizootic haematopoietic necrosis (EHN)	Epizootic haematopoietic necrosis virus
Infectious haematopoietic necrosis (IHN)	Infectious haematopoietic necrosis virus
Oncorhynchus masou virus disease	*Oncorhynchus masou* virus (OMV)
Spring viraemia of carp	Spring viraemia of carp virus (SVCV)
Viral haemorrhagic septicaemia (VHS)	Viral haemorrhagic septicaemia virus
Channel catfish virus disease	Channel catfish virus (CCV)
Viral encephalopathy and retinopathy	Striped jack nervous necrosis virus (SJNNV)
Infectious pancreatic necrosis	Infectious pancreatic necrosis virus (IPNV)
Infectious salmon anaemia	Infectious salmon anaemia virus (ISA virus)
Epizootic ulcerative syndrome	*Aphanomyces invaderis* (fungus)
Bacterial kidney disease	*Renibacterium salmoninarum* (bacterium)
Enteric septicemia of catfish (ESC)	*Edwardsiella ictaluri* (bacterium)
Piscirickettsiosis	*Piscirickettsia salmonis* (rickettsia)
Significant diseases of bivalve mollusks	
Bonamiosis	*Bonamia* spp. (protozoan)
Haplosporidiosis	*Haplosporidiosis* spp. (protozoan)
Marteiliosis	*Marteilia* spp. (protozoan)
Mikrocytosis	*Mikrocytos* (protozoan)
Perkinsosis	*Perkinsus* spp. (protozoan)
Iridoviroses	Iridoviruses
Significant diseases of crustaceans	
Baculoviral midgut gland necrosis virus	Baculoviral midgut gland necrosis (BMN)
Nuclear polyhedrosis baculoviroses	Baculoviruses
Infectious hypodermal and haematopoietic virus	Infectious hypodermal and haematopoietic necrosis virus (IHHN)
Yellowhead disease	Yellowhead virus (YHV)
Crayfish plague	*Aphanomyces astaci* (fungus)

[a] Notifiable diseases are those that are of economic importance and of widespread geographical distribution.

Other International Animal Health Organizations

The OIE has working relationships with the World Health Organization (WHO) and Food and Agriculture Organization (FAO), plus the World Trade Organization (WTO), the Inter-American Institute for Cooperation on Agriculture (IICA) and the Pan American Health Organization (PAHO). The WHO has an interest in animal health because of the impact on human health. The FAO, an agency within the United Nations, has a mandate to improve agricultural productivity. The FAO also provides policy and planning advice to governments. Besides the Fish Health Section of the American Fisheries Society, other international professional organizations involved with aquatic animal health include the Asian Fisheries Society, World Aquaculture Society, European Association of Fish Pathologists, International Association for Aquatic Animal Medicine, Japanese Society of Fish Pathology and National Shellfisheries Association. The American Veterinary Medical Association (AVMA), U.S. Animal Health Association (USAHA), and American Association of Veterinary Laboratory

Diagnosticians (AAVLD) also direct their attention to aquatic animal health with respect to decreasing the occurrence of disease, to standardizing diagnostic laboratory tests and to communicating and coordinating regulatory policies.

Code of Federal Regulations: Title 50 -- Wildlife and Fisheries

The Code of Federal Regulations (CFR) is a codification of the rules published by the Office of the Federal Register, National Archives and Records Administration. Title 50 (Wildlife and Fisheries) Chapter I (U.S. Fish and Wildlife Service, Department of the Interior) Part 16 (Injurious Wildlife) Subpart B (Importation or Shipment of Injurious Wildlife) Section 16.13 (Importation of Live or Dead Fish, Molluscs and Crustaceans, or their Gametes or Fertilized Eggs) of the CFR (1997) offers very specific guidelines on fish pathogens in the family Salmonidae. The guidelines are more general with regard to other fish species. As specified in Title 50, salmonid gametes to be shipped into the United States must be accompanied by a certification performed by a Title 50-certified fish pathologist from their country of origin.

The Director of the U.S. Fish and Wildlife Service may enter into formal agreements that allow the importation of live fish if the exporting nation has an acceptable program of inspection and pathogen control in operation, can document the occurrence and distribution of fish pathogens within its boundaries and can demonstrate that importation of the fishes will not pose a substantial risk to the public and private fish stocks of the United States (CFR 1997). However, this program has not been implemented to date. Live fish have been imported from Canada on a case-by-case basis when it has been demonstrated that they will not transmit controlled pathogens and this is acceptable to the accepting state. The CFR states that no progeny or eggs of imported species may be released into the wild except by the state wildlife conservation agency having jurisdiction over the area of release or by persons having prior written permission from such agency.

Additionally, the CFR provides guidelines for specific sampling requirements for shipments of gametes and fish and for methodologies on virus assays. General sample processing requirements list concentrations and types of antibiotics and antifungal agents that may be added to tissue samples to control microbial contaminant growth at the time of sample collection. The CFR also specifies what is required in the certificate to accompany importations. Information concerning particulars of importation and certification requirements and state fisheries resources contacts can be obtained at the U.S. Department of the Interior, U.S. Fish and Wildlife Service, Division of Fish Hatcheries, 4401 North Fairfax Drive, Arlington, Virginia 22203.

National Fish Health Policy of the U.S. Fish and Wildlife Service

Fish pathogens are disseminated by migratory and anadromous fish, the improper disposal of offal, the illegal transfer or accidental introduction of live fish, the movement of commercially caught fish for processing, the transport of fish for establishing recreational fisheries or aquaculture, or as part of the ornamental fish trade. The responsibility for managing fishery resources in the United States lies with the individual states. The USFWS Fish Health Policy (USFWS 1995) serves as the basis for the Service’s effort to restrict disease movement or introduction and to minimize the impacts of fish pathogens and diseases within the National Fish Hatchery System (USFWS 1995). Infected eggs (e.g. eggs that have not been surface disinfected) or mature fish can be moved into different habitats prior to the development of a specific pathogen detection technique, or before the pathogen is recognized (Fryer and Bartholomew 1996). The Fish Health Policy is a model for freshwater and marine fisheries efforts. Gametes,

fertilized eggs or fish are shipped or accepted in compliance with the USFWS Policy, area disease control programs, regional stock transfer policies and state regulations.

A USFWS pathogen coding system summarizes results of inspections and regular monitoring of Service facilities and fish stocks (USFWS 1995). Gamete samples from fish that are known to be infected are to be received only by hatcheries that have the appropriate pathogen designation code. For specifically named pathogens, the USFWS Policy provides rules and time periods for transferring fish and eggs. Fish Health Inspection Reports (see attachment this chapter) mandate provisions for documenting the presence of additional pathogens. The Fish Health Policy specifies the methodology to be followed during inspections for bacteria and parasites, as outlined by the latest edition of the Fish Health Blue Book. The Blue Book provides suggested procedures for the detection and identification of certain finfish and shellfish pathogens and is published through the Fish Health Section of the American Fisheries Society (Thoesen 1997). The policy provides specific guidelines for reducing risks of pathogen transfer with respect to the transfer or handling of gametes. All translocations of cryopreserved sperm could use a similar coding system that summarizes results of facility and stock inspections. A program carried out at USFWS hatcheries for the cryopreservation of fish sperm would necessitate working with the provisions of this policy.

Great Lakes Fishery Commission

Fish disease control in the Great Lakes basin is managed by the Great Lakes Fish Disease Control Committee of the Great Lakes Fishery Commission (GLFC). The GLFC has devised a control policy and model program (Hnath 1993) that sets forth requirements for the prevention and control of infectious diseases, and a protocol that proposes recommendations to minimize the risk of introducing disease agents with the importation of salmonid fishes (Horner and Eshenroder 1993). The control policy encourages agencies to work together to control fish diseases, and coordinates their fish management programs. Categories of infectious disease and classifications of salmonid fish hatcheries are based upon disease history. Measures to minimize the spread and introduction of disease agents, including detection and treatments, are provided (Hnath 1993). The protocol provides guidelines to be followed by the member agencies who represent private and government sectors concerned with aquaculture and fisheries in the Great Lakes basin. The Commission aids in the development of legislation and regulations involved in minimizing the risks associated with introducing disease agents with imports of salmonids.

Just as diseases or disease agents of importance to artificial insemination and embryo transfer in livestock have been grouped and categorized regarding the known risks of transmission (Thacker et al. 1984, Philpott 1993), similar categories might be established for use with aquatic species as are designated in the control policy of the GLFC (Hnath 1993). As pointed out above, a pathogen coding system, described in the National Fish Health Policy (USFWS 1995), designates broodstock facilities and fish stocks according to the presence of the specific disease agents listed in the policy. A cryopreservation program can make use of such designation systems.

Animal Plant Health and Inspection Services

Veterinary Services

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture provides leadership to ensure the health and care of animals and plants, to improve agricultural productivity and competitiveness and to contribute to the national economy and the

public health. The Veterinary Services (VS) arm of APHIS protects and improves the health, quality and marketability of U.S. animals and animal products by diagnosing, preventing and controlling animal diseases, monitoring for new threats, and responding to emergencies. Veterinary Services assists the aquaculture industry by issuing export health certifications for live fish and fish eggs and by ensuring the veterinary biological products are safe, pure, potent and effective. While APHIS is authorized to prevent the introduction and dissemination of pests and diseases that can harm U.S. livestock and crops, it does not have authority to take similar actions to protect the aquaculture industry from foreign pests and diseases. The aquaculture contact can be reached at USDA, APHIS, VS, 4700 River Road, Unit 43, Riverdale, Maryland 20737-1231.

International Services

This is a subunit of APHIS that represents the U.S. government in dealing with international and regional organizations concerned with animal and plant health. APHIS has found that the successful exclusion of exotic pests and diseases from the United States depends heavily on the cooperation of counterpart agencies in other countries, and exclusion is preferable to control after establishment. Therefore, International Services cooperates in a number of surveillance and control programs in foreign countries to reduce the threat to agriculture in both countries, and works closely with OIE.

Permitting Requirements

At the outset of a cryopreservation program, cooperation among and within government agencies and with scientific institutions regarding permitting requirements is required. Materials derived from all animals are potentially subject to USDA regulations and must be cleared by their inspectors at the port of arrival before entry into the U.S. is authorized. Importation of amphibians, fish, reptiles, shellfish and aquatic species, including their blood, tissues, serum, feces, extracts, fluids, venom and urine requires permits. Importation of livestock, as well as their semen or embryos, requires health certification issued by an official of the exporting country. The USFWS Office of Management Authority at 4401 North Fairfax Drive, Arlington, Virginia 22203 and USDA, APHIS, 4700 River Road, Riverdale, Maryland 20737 can be contacted for specific information on health certifications.

Permits from the USDA are required for import of any microorganism that is known to cause infectious, contagious or communicable diseases of livestock or poultry. Non-pathogenic microorganisms, including environmental or water organisms, may be imported into the country without USDA veterinary restrictions. Consideration of shipping microorganisms as reference specimens may be pertinent to a cryopreservation program. Specific guidelines for labeling, packaging and shipping of microorganisms internationally may be obtained from the American Society for Microbiology, 1325 Massachusetts Avenue, Washington, D.C. 20005.

National Aquatic Animal Health Management Program

In view of the varying regulatory requirements for fisheries management in the United States, the Joint Subcommittee on Aquaculture attempted to develop a national strategy. This effort was carried on in 1997 by the Aquatic Animal Health Policy Development Committee that had been established by the National Association of State Aquaculture Coordinators and the National Aquaculture Association. Its objective is to develop and to promote a coherent national aquatic animal health management program that serves commercial aquaculture interests (National Fish Health Research Laboratory 1998). A framework has been established for drafting

a national policy that outlines procedures for future aquatic animal health management decisions. Various international, national and state aquatic animal health programs and authorities will be involved.

Data Management

A sophisticated approach for managing and summarizing data on gamete collection, storage and dissemination should be taken at the outset of any repository program. A gamete repository with archival samples is useful for a number of reasons including examining the role of pathogens in ecosystems (O'Brien and Evermann 1988). Additionally, epidemiologists may need to trace the pathway of an infectious agent, molecular biologists may need to evaluate homology of genetic material to develop probes, or wildlife officials may need comparative material to establish genetic variation of populations (Sheldon and Dittman 1997). In this regard, the responsibilities of a gamete repository would include good record-keeping and organization and fast delivery of samples and paperwork to researchers.

A documented quality assurance system that includes data management practices is crucial for an archival program in cryopreserved gametes from aquatic species. Independent authorities could inspect and oversee the tracking of information and establishment of a controlled quality assurance policy and provide maximum reliability of samples (Kincaid, this volume). Traditional and innovative systematic collection methods could be employed. The following illustrates the necessary components of an archival program in cryopreservation of gametes from aquatic species:

Record-keeping

The information recorded for a collection is critical to the future use of archival material (Rivers and Ardren 1998). Minimally, information should include collection date, species, exact sampling location, method of collection, preparation method and people associated with contributing the sample. Users should be able to locate samples quickly, so a record of storage sites is needed. An accessible and comprehensive database would facilitate immediate access to information on available gamete samples, broodstock from which they were derived and associated microbiological concerns. A centralized database could receive data electronically from multiple sites and would therefore contain updated information on surveillance activities. A centralized location for information could also offer suggested protocols for use of gametes and for proactive

United States Department of the Interior
U.S. Fish and Wildlife Service
FISH HEALTH INSPECTION REPORT

This report is **not** evidence of future disease status. To determine current status, contact inspecting biologist below.

Name of Fish Source	**Location of Fish Source**	**Name of Owner or Manager**

Inspection (Date)	**Classification**	**Fish Examined**	**Water Supply**	
		☐ **Hatchery** ☐ **Salmonid** ☐ **Non-Salmonid** ☐ **Feral**	☐ **Spring** ☐ **Stream** ☐ **Well**	☐ **Impoundment** ☐ **Enclosed** ☐ **Free of fish**

Species[1]	**Lot Number**	**Age**[2]	**Number in lot**	**Obtained as Eggs(E) or Fish(F) FROM:**	**Pathogens inspected for and Results**[3]									
					IPN	**IHN**	**VHS**	**OMV**	**RS**	**AS**	**YR**	**WD**	**X**	**Y**

Remarks:

Inspecting Biologist Address	Inspecting Biologist Signature
Phone Fax	Name:

[1] Use standard FWS abbreviations (see back of this page)
[2] Hatchery fish give age in months: feral fish use symbols E = eggs or fry; F = fingerlings; Y = yearlings; B = older fish
[3] See list of pathogen abbreviations on back of page; findings reported $\frac{\text{Number examined}}{\text{Results}}$ where += positive and - = negative; other pathogens listed in remarks.

INSPECTION INFORMATION CONTINUED

Species[1]	Lot Number	Age[2]	Number in lot	Obtained as Eggs(E) or Fish(F) FROM:	Pathogens inspected for and.									
					IPN	IHN	VHS	OMV	RS	AS	YR	WD	X	Y

SUPPLEMENTAL INSPECTION INFORMATION

Date	Species	Lot#	Findings

PATHOGEN ABBREVIATIONS

AS	Aeromonas salmonicida
YR	Yersinia rucken
RS	Renibacterium salmoninarum
IPN	Infectious pancreatic Necrosis Virus
IHN	Infectious Hermatopoietic Necrosis Virus
VHS	Viral Hemorrhagic Seticemia Virus
OMV	Oncorthynchus masou Virus
WD	Myxobolis cerebralis
A	No listed pathogens detected
SPF	Specific Pathogern Free
S	Suspect Designation
X	Other- see remarks
Y	Other- see remarks

SPECIES ABBREVIATION

ARG	Arctic Grayling
ATS	Atlantic Salmon
BCF	Blue Catfish
BKT	Brook Trout
BNT	Brown Trout
CCF	Channel catfish
CHS	Chum Salmon
COS	Coho Salmon
CUT	Cutthroat Trout
DAR	Darters
DOV	Dolly Varden
FCS	Fall Chinock Salmon
GIT	Gila trout
GOT	Golden trout
GRC	Grass Carp
LAS	Landlocked ATS
LAT	Lake Trout
OHT	Ohrid Trout
OSA	Other Salmon
PAH	Paddlefish
PKS	Pink Salmon
RBT	Rainbow Trout
RBTSST	RainbowXSteelhead
SCS	Spring Chinock Salmon
SOS	Sockeye Salmon
STB	Striped bass
SSN	Shortnose Sturgeon
STN	Sturgeons
STT	Steelhead Trout
WCS	Winter Chinock Salmon

Remarks:

Form-3-226(7/97)

measures regarding microbiological concerns. Appropriate references would be listed for specific analytical procedures.

The National Fish Health Policy specifies the completion of Fish Health Inspection Reports by fish health biologists for submission to fish hatcheries. The policy states that inspection reports will be maintained on the disease status of all federal, state, tribal and commercial fish facilities and wild fish stocks. This form, along with the model international health certificates found in the OIE Fish Diseases Commission International Aquatic Animal Health Code (1997b), plus the certificate used to accompany fish imports (CFR 1997) can be used to fashion appropriate certificates for use with cryopreserved materials.

The National Plant Germplasm System, coordinated by the Agricultural Research Service of the U.S. Department of Agriculture, consists of well-organized collection of reference specimens of numerous species. Each collection has an associated library that documents specimen hosts, geographic distribution and other useful information. Because these collections are useful working tools and are recognized universally for their contribution to research, this system is a model for an efficient archival network.

Surveillance

A surveillance system that documents incidences of individual disease organisms in hatchery animals, wild fish and laboratory samples would measure any impact of fish produced from cryopreserved gametes, allow evaluation of the effectiveness of prevention measures taken during cryopreservation methods and enable tracing of unusual occurances. Reliable surveillance data provides baseline information on incidence trends and geographic distribution of known infectious agents. Surveillance is the single most important tool for identifying and monitoring infectious diseases and the effectiveness of regulations can only be adequately assessed if effective surveillance systems are in place (National Center for Infectious Diseases 1994).

Sentinel Animals

The use of sentinel animals to enhance surveillance of human diseases has been an effective public health tool, and might be considered for use with fish produced with cryopreserved gametes having questionable disease agent status. Such enhanced monitoring and data collection might become routine for those partners using cryopreserved gametes.

Risk Analysis

A strategic modeling approach might be employed to provide practical insight when the current state of knowledge is insufficient for risk analysis or when general or global questions arise that cross disciplinary boundaries (Puccia et al. 1994). The use of a mathematical model integrated into a program at the outset might benefit this new area.

If necessary, susceptibility testing of fish gametes or stocks to specific pathogens could be performed in order to evaluate risk. That would ensure that the gametes or stocks would be tested for deleterious effects prior to initiating cryopreservation efforts. Co-habitation experiments under laboratory conditions could measure the potential for direct horizontal transmission. The dual importance of pathogen virulence and the transmission rate of the agent in host populations is primary in these equations.

Risks of introduction must be considered and identified and the at-risk populations protected. Understanding the pathogenesis of microbial pathogens will result in more informed health (Thune et al.1993, Hedrick 1998) and risk management decisions. Assessing the potential risk due to the use of contaminated gametes, whether in disease transmission or in lowered

quality of sperm, is dependent upon knowledge of the factors that influence disease occurrence or sample degradation. Some specific questions to ask are: what is the host range of the pathogen? Is it likely that the pathogen has the potential to proliferate and establish infection in the particular system? Is it likely that pathogen transmission would occur under the conditions being employed?

Emergence of Disease

Possible emergence of diseases by the use of cryopreserved sperm would be driven by some of the same factors contributing to the emergence of food-borne and other infectious diseases such as: technology and industry, international travel and commerce, microbial adaptation, economic development and global environmental changes (Morse 1993, Wilson 1994, Altekruse et al.1997). Emerging infections can be defined as infections that are either newly appearing in a population, rapidly increasing their incidence, or expanding their geographic range (Morse 1993). Conditions that allow the exposure of previously restricted microbes to new host populations are important in the emergence of disease. Most new infections are not caused by novel pathogens (Wilson 1994). Processing sperm samples could be viewed as a step that amplifies the occurrence of disease agents.

Emerging diseases are a major concern for aquatic animal health worldwide. Emerging microbial pathogens in fish share characteristics such as: poor or no response to therapy, existence in asymptomatic carriers, ability to establish themselves in wild fish populations, causation of significant economic and ecologic losses and horizontal and vertical transmission despite topical egg disinfection (Hedrick 1998). An example of an emerging infectious threat to aquatic animals is the Central American shrimp viruses transported in 1994-1995 to Texas coast shrimp facilities (Carlton 1996). Gathering information about the numerous factors that effect disease emergence, such as potential reservoirs or vectors, is important (National Center for Infectious Diseases 1994). Controlling these agents often relies on effective regulatory approaches (Hedrick 1998).

The presence of exotic pathogens should be considered dangerous and a possible source of infection in the laboratory and for all samples passing through it. If a pathogen is detected, the assumption can be made that other fish will be at an increased level of risk for infection, so proactive actions should be taken. However, detecting a pathogen does not indicate it is clinically significant. Several disease agents are ubiquitous in nature, such as those causing infectious pancreatic necrosis (IHN), furunculosis and vibriosis. These are included among the fish pathogens inspected for at USFWS facilities. Inspected pathogens include *Aeromonas salmonicida*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, viral hemorrhagic septicemia virus, infectious pancreatic necrosis virus (IPNV), IHNV and *Oncorhynchus masou* virus (USFWS 1995).

Pathogen transmission between agricultural and wild species has been reported. Examples include avian influenza and fowl cholera between poultry and waterfowl, brucellosis between domestic and feral swine and foot-and-mouth disease between cattle and wild ruminants. Microorganisms sometimes escape even from secure environments, so pathogen transmission must be considered possible. Additionally, despite the most vigorous testing of sperm samples, transmission of infectious disease is possible (Zilberstein et al. 1997).

Conclusions

When establishing a large-scale program in cryopreservation of gametes from aquatic species, many variables need to be considered, such as economic decisions, priority species for conservation and limiting microbe transmission. The foundation of an effective, large-scale, well-designed program for cryopreservation in aquatic species would include a clear definition of goals and objectives, an understanding of the limitations of the methodologies for pathogen detection, and the consideration of consequent risks. Moreover, the program should provide a comprehensive strategy for the collection and storage of good quality gametes free of microbial threats.

National and international uniformity of policies and regulations would facilitate the development and operation of a science-based cryopreservation program. Program development should take advantage of the historical delineations of health management responsibilities in existing regional, national and international programs. A number of policies and regulations, such as the Great Lakes fish disease control policy and model program (Hnath 1993), National Fish Health Policy (USFWS 1995), CFR (1997) and OIE Fish Diseases Commission International Aquatic Animal Health Code (1997b) will be useful as models for an archival program. Improvements in diagnostic techniques will allow greater precision in confirming the presence or absence of microbes of concern, and greater confidence for international transport of gametes.

A high integrity program will ensure that animal and ecosystem health is not compromised. Coordination is critical to the success of a global gamete cryopreservation program. Properly instituted policies relating to data management, ownership and access rights to genetic resources, national and international technology transfer and disease prevention will build a stable framework for the future success of such a program. A strong national fish health program will be necessary to establish and maintain both domestic and international trade.

Internationally approved standards of collection, processing, and distribution of cryopreserved material from aquatic species are essential to ensure quality and freedom from disease. Knowledge of factors affecting the distribution of particular diseases and the mechanisms by which pathogens perpetuate in nature is essential for establishing policies for the importation of live animals and germplasm (Gibbs 1981). When there is an understanding of the connections among ecosystem structure and function, the health of aquatic species, and the risks involved with transported gametes or species, informed decisions can be made concerning the use of cryopreserved gametes and embryos from aquatic species for resource management and aquaculture practices.

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New Chapter

The Relationship Between Conservation Policy and Aquatic Genetic Resources

Jennifer Long and Harvey D. Blackburn

Introduction

Globally there is an increased awareness of how important genetic resources are for all forms of agriculture. This awareness has resulted in questions concerning the policies that specific countries and the international community should pursue to ensure the sustainable use of diverse genetic resources. For much of the agricultural sector national policies are of interest because of a general contraction in the amount of genetic diversity available for developing new varieties or lines that can increase agricultural productivity. The Food and Agriculture Organization of the United Nations (2006) reports that marine and inland aquaculture have exhibited significant growth in the quantity of production (Figure 1). During the last 30 yr and across the species categories crustaceans have generally had the fastest growth rate, however all categories have hold a 5% or greater growth rate indicating production is expanding at exponential rates.

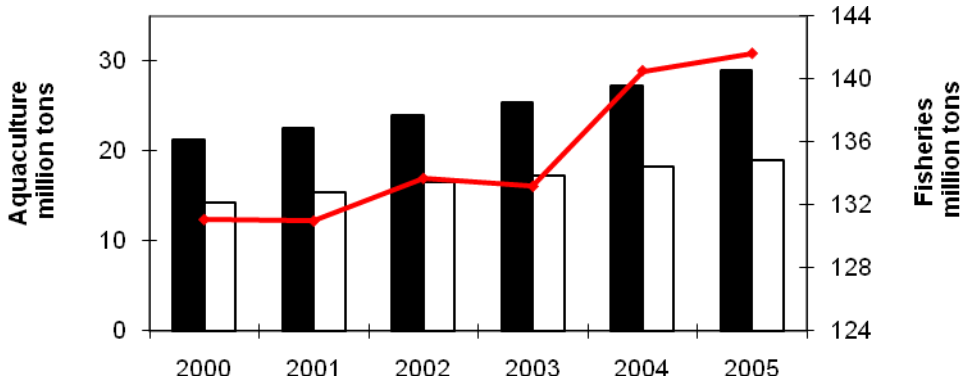

Figure 1. World production from inland (black bars) and marine (white bars) aquaculture (left axis) vs. total world fisheries (dotted line) (right axis).

From 2000 to 2004 value of aquaculture has grown by 23% to US$71.5 billion (FAO, 2006) (Figure 2). With this increase in production and value fish are widely traded, it is estimated that 38% (live weight equivalent) is exported. In 2004, regions showing a trade surplus included Africa, Latin America and the Caribbean, and Oceania; while those reporting a deficit included Asia, North America, and Europe (FAO, 2006).

As pointed out by FAO (2006) the production increases achieved to date have not been the result of genetic selection, rather they have resulted from improvements in nutrition, management, and increased area of production. This type of sector development is typical of other components within agriculture. For example, increased production in milk due to genetic selection did not have a significant impact on dairy production until about 1970 after quality of management and nutrition had increased. Aquaculture may follow this same developmental pattern. A key difference between aquatic species and other forms of animal agriculture is that aquaculture still has the ability to capture animals from the wild and incorporate those genetics

Long, J. and H. D. Blackburn. 2011. The Relationship between Conservation Policy and Aquatic Genetic Resources. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 977-983.

into captive breeding populations. As long as captive populations have not been intensively selected for production characteristics the introduction of wild genetics may be useful. However, once selection intensity increases and captive populations become more genetically divergent from their wild progenitors the value of such introductions will be limited. What must also be considered in the use of genetic variability of aquatic species is that selection can be more intensively practiced than in other species due to the ability to propagate populations from a small number of parents. As a result, the genetic diversity in aquatic populations can rapidly decrease.

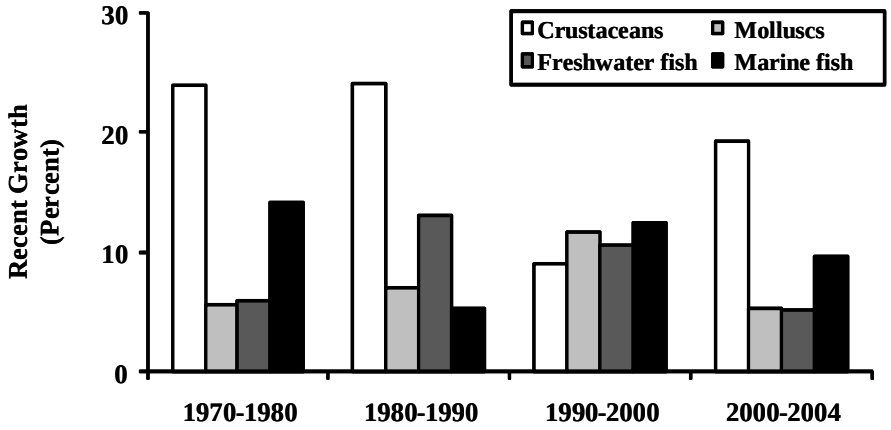

Figure 2. World aquaculture production: average annual growth rate for different species groups.

Compared to livestock or crop agriculture the scale of international exchange of aquatic genetic resources is limited. There are a few key species where active trade exists. The most notable example is the shrimp industry, which obtains its genetic resources from a number of geographic locations, from which it develops specific lines, which in turn are sold globally. As selective breeding becomes more prevalent in aquaculture a similar type of model may emerge for other species. For this reason understanding the current policy landscape dealing with genetic resources will be of utility as aquaculture continues to develop and the exchange of genetic resources grows. It is this issue in conjunction with the movement and exchange of genetic resources of aquatic animal and the relationship to the current discussions concerning the international use and exchange of genetic resources which we wish to discuss in this chapter.

The Policy Landscape

Depending on the species of interest to commercial or subsistence-level producers, access to aquatic animal germplasm occurs along a continuum of formal to informal systems – ranging from well-established private sector channels to the acquisition of wild stocks directly from natural areas. The diversity of stakeholders and systems for managing and using aquatic genetic resources necessitates that there be information exchange between aquaculture stakeholders and the negotiators representing them at the international fora in which these issues are being examined. Given the potential impact on global aquaculture of an international regime intended to manage access to and benefit sharing from the use of genetic resources, further examination of how current systems are operating could assist in the development of a regime that facilitates the conservation and sustainable use of these critical resources. The aquaculture community would benefit from engaging in deliberations of how to best inform the development of emerging

international legal frameworks on access and benefit sharing issues. These emerging approaches could take a number of forms, ranging from little or no government intervention to highly regulated exchanges requiring strict contractual arrangements between private parties or between governments.

Table 1. International organizations involved with genetic resources.

Organization	Activities relevant to genetic resources
Convention of Biological Diversity (CBD)	Among the CBD's objectives are "the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources…" (CBD, 1992). Article 15 of the Convention is focused on the issue of access to genetic resources and in 2000, the CBD created an ABS (Access and Benefit Sharing) working group to "develop guidelines… to assist … with the implementation of the access and benefit-sharing provisions of the Convention" (CBD, 2002). By 2010, the CBD has tasked its ABS Working Group to develop an approach that accommodates the needs and interests of a range of stakeholders across many sectors.
World Trade Organization Trade-Related Aspects of Intellectual Property Rights (WTO TRIPS)	Article 27 of the TRIPS agreement relates to patenting. Paragraph 19 of the 2001 Doha Declaration indicates that the TRIPS Council should examine the relationship between the TRIPS Agreement and the CBD – for instance, to examine issues such as the inclusion of the origins of genetic materials in patent applications (WTO 2008a, b).
United Nations Convention on the Law of the Sea (UNCLOS) and United Nation (UN) Fish Stocks Agreement	UNCLOS is the principal forum for the discussion of marine genetic resources in international waters, as discussed at the Consultative Process Meeting in June 2007, although UNCLOS also deliberates marine genetic resource access and benefit-sharing issues within national jurisdictions. Marine genetic resources, as discussed in the Consultative Process Meeting, primarily refer to microbial organisms found in marine environments while fish genetic resources are indirectly addressed in the UN Fish Stocks Agreement.
Commission on Genetic Resources for Food and Agriculture (CGRFA)	Addresses policy and technical aspects of genetic resource issues for all agriculturally important areas (crops, livestock, forestry, aquaculture) and capture fisheries, microbes, and insects. Responsible for the development of the International Treaty on Plant Genetic Resources. Provides guidance to FAO on activities in this area.
World Intellectual Property Organization (WIPO)	Works generally on intellectual property (IP) aspects of genetic resource access and benefit-sharing issues (WIPO 2008).

There are several pre-existing intergovernmental fora (Table 1) that produce internationally legally binding arrangements that impact all of agriculture. For example, the World Trade Organization and their efforts in Trade-Related Aspects of Intellectual Property

Rights (WTO TRIPS); the Convention of Biological Diversity (CBD); and the United Nations Convention on the Law of the Sea (UNCLOS). All of these bodies deliberate on issues that impact the aquaculture community. Perhaps more relevant to agriculture and aquaculture, in general, are the ongoing efforts at the United Nations (UN) Food and Agriculture Organization (FAO). Specifically, the Aquaculture and Fisheries Department of FAO has served as secretariat for member countries in developing the Code of Conduct for Responsible Fisheries. Of late, the Commission on Genetic Resources for Food and Agriculture (CGRFA) has added activities on aquatic genetic resources in its multi-year plan of work and in conjunction with its efforts on plants and livestock. The CGRFA may play an important role in developing arrangements concerning aquatic genetic resources because the CBD, the central forum for negotiations on access and benefit sharing (ABS) from the use of genetic resources, has turned to the CGRFA to contribute its expertise on how benefit sharing for genetic resources relevant to food and agriculture may be best approached.

Much of the rationale for the focus of the CBD has been an emphasis on wild populations and concerns about the use of various genetic resources as they relate to the pharmaceutical industry. It does not appear that this model is particularly effective for agriculture in general. For as illustrated by Gollin et al. (2008), the flow of livestock genetic resources from non- OECD (Organization for Economic Cooperation and Development) countries to OECD countries is quantitatively and economically small. For plant genetic resources of relevance to food security, the International Treaty on Plant Genetic Resources for Food and Agriculture, adopted in 2001, was developed to govern the exchange of these resources, of which many are maintained in *ex situ* collections developed long before the CBD entered into force. Given the diversity of exchange and use patterns of genetic resources across agricultural sectors, the CBD has requested support from the CGRFA.

Management of Aquatic Genetic Resources

The future development of aquatic genetic resources for aquaculture will depend upon private sector interest and development of specific populations that confer an advantage to production. To date and where genetic selection is applied, aquaculture has been successful in developing markets for such populations. Intellectual property rights or other forms of exclusive ownership or access have rarely been sought or enforced to date for farmed fish. At issue will be to what degree benefit sharing has relevance to the market place and to what extent the CBD or an emergent benefit sharing agreement play a role in the maintenance, utilization and conservation of aquatic genetic resources used in aquaculture. While national sovereignty over wild fish populations is provided by the CBD, arguments can be made for recognition of new countries of origin for strains, hybrids or other forms of alien fish species that have acquired distinctive properties by being farmed outside their native ranges (Pullin 2005).

To date there are two widely different models of how to proceed in conserving and fostering the utilization of genetic resources. One example is the almost two decades of work that was put into developing the International Treaty on Plant Genetic Resources for Food and Agriculture. Under this agreement, specified plant genetic resources from contracting parties have been made part of a multilateral system. Those wishing to use genetic resources from another country must adhere to the terms of an internationally agreed upon standardized material transfer agreement. The terms of access and benefit sharing have been defined in the ITPGRFA. While the development of a treaty and material transfer agreement have been seen by some as a

positive step, to date the ITPGRFA seems to have reduced the exchange of plant genetic resources between countries. At the other end of the spectrum is the livestock sector, which has strong market structure in place to handle the exchange of genetic resources. Gollin et al. (2008) suggest that for the livestock sector there is little basis for the type of formal agreements that have been developed by the plant community. Pullin (2005) articulates the similarities between aquatic and livestock genetic industries in that companies produce elite lines and hybrids and then tend to distribute their products to farmers through commercial agreements, (e.g., contract growing).

The aquaculture sector has addressed several aspects of germplasm exchange. In the Code of Conduct for Responsible Fisheries of the FAO it is recommended that states conserve genetic diversity and maintain integrity of aquatic communities. In addition, states should encourage adoption of appropriate practices in genetic improvement of broodstocks to minimize disease transfer. The Nairobi Declaration on the use of genetically improved and alien species in Africa (ICLARM, 2002), recognizes that:

1. Captive breeding populations can lose genetic diversity and therefore such considerations should be a basic element in broodstock management.
2. That unique wild stocks of tilapia in Africa need identification and protection from introductions of genetically improved strains.
3. Access to baseline information on fish genetic diversity needs to be strengthened.

Based upon the FAO Code of Conduct for Responsible Fisheries and the Nairobi Declaration, the WorldFish Center (www.worldfishcenter.org) has developed a policy and protocol for the transfer of Genetically Improved Farmed Tilapia. While this material transfer agreement is focused upon exchanges from WorldFish Center to governments it does suggest that the necessary elements for exchanging germplasm are in place and functioning from a public sector perspective.

The Use of Aquatic Genetic Resources

In the coming years, global aquaculture industries may increasingly devote attention to broodstock development, and reliance on access to wild materials will likely decrease as broodstock improvement programs mature. In the United States, hybrid striped bass broodstock producers obtain breeding materials from wild sources on an annual basis. These breeding programs are still in early stages of development and it is likely that as these programs mature, use of improved populations for broodstock development will decrease the need for wild stocks. Conversely, the US rainbow trout industry no longer obtains wild animals for breeding as they have well-established breeding populations with ample genetic variability.

Until global aquaculture achieves a level of sophistication that no longer requires access to wild stocks for routine breeding activities, *ex situ* collections may be useful in the coming years. For example, access to *ex situ* collections may facilitate progress in breeding for specific traits of interest through identification of useful genes in wild populations that may exist in lower frequencies in advanced breeding populations.

Current trends in the development of cryopreservation and other aquatic animal germplasm storage techniques will facilitate the development of these collections. Over the next few decades, the development of international legal frameworks that govern the terms of access to and use of aquatic animal genetic resources will coincide with the maturation of global aquaculture broodstock development and the development of *ex situ* germplasm collections.

Therefore, although more detailed agreements have yet to be established, those involved in active germplasm acquisition for either breeding programs or *ex situ* collections need to understand the terms by which these materials can be obtained from source countries. Currently, guidance on this issue has been outlined in the CBD text which states that obtaining materials requires prior informed consent and mutually agreed terms. Identifying the appropriate competent authorities with whom to establish informed consent and mutually agreed terms can be complicated with aquatic organism germplasm, particularly in transboundary aquatic ecosystems where waters reside in multiple jurisdictions.

Policy Considerations and Conclusions

Management and utilization of aquatic genetic resources will require access and utilization of populations that have been developed to meet various production system needs throughout the world. Public and private sector initiatives have been developed to meet market needs. Where there are viable markets and exchange of aquatic germplasm, the issue at hand becomes what sort of policies are needed to protect genetic diversity and ensure that the aquaculture sector can continue to develop and contribute to the nutritional and economic well-being of people.

As previously stated, aquaculture has the ability to rapidly reduce the genetic variation in aquatic populations due to short generation intervals and high reproductive rates. In addition there is a need to offer appropriate levels of protection to wild populations so they can be used to contribute to industry development. This issue can be addressed at the country level by the inception of national programs (which include establishment of cryopreserved collections) to assist in the management of aquatic genetic resources. A component of such a national effort is the development of a gene bank capable of storing cryopreserved samples of species a country deems important. In addition such programs with their cryopreserved collections can facilitate the CBD obligations a country may have.

Another element of national program is the development of baseline information concerning the status of the appropriate aquatic genetic resources, as has been articulated in the Nairobi Declaration. In addition to policies on conservation there is a critical need to develop and implement policies that address: incentives (excluding subsidies) to productoin, access to markets for outputs and inputs, and facilitation of integration of conservation, demand and environmental elements of aquaculture production (Norton 2004, Blackburn 2007). As with other life forms, access and benefit sharing will be of interest to the aquaculture sector. It is becoming apparent from discussions in the livestock sector that for any type of formal or informal arrangement there is a need for agreements to be flexible and to facilitate the development of the sector and not impinge upon its ability to provided needed food and economic activity.

The global agriculture community will have to contend with contracting genetic diversity, the ability to introduce (introgress) genes of interest into productive populations, and an increased awareness about the public and private rights associated with these resources. In this arena aquaculture has special challenges due to the ability to access and utilize wild populations, and the vast array of life forms used to feed a hungry world. While the production challenges may be significant, the multilateral agreements and initiatives discussed in this chapter clearly demand attention of the aquaculture sector to insure that effective and rational agreements are developed. This will require aquaculture representatives to quickly become familiar with the

topic and to gain long-term understanding as to where the industry and its sub-sectors are headed. Only then will policy makers be in a position to effectively develop the needed policies for sustainable and beneficial use of aquatic genetic resources.

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Ethical Considerations for the Application of Cryopreservation to Aquatic Species

Stephen S. Wachtel and Terrence R. Tiersch

"All things considered it looks as though Utopia were far closer to us than anyone ... could have imagined ... Indeed, unless we choose to decentralize and to use applied science, not as the end to which human beings are to be made the means, but as the means to producing a race of free individuals, we have only two alternatives to choose from: either a number of national, militarized totalitarianisms, having as their root the terror of the atomic bomb and as their consequence the destruction of civilization (or, if the warfare is limited, the perpetuation of militarism); ... or else one supra-national totalitarianism, called into existence by the social chaos resulting from rapid technological progress in general and the atomic bomb in particular, and developing, under the need for efficiency and stability, into the welfare-tyranny of Utopia. You pays your money and you takes your chances."

Aldous Huxley (1894-1963)
Foreword to Brave New World (1946)

Introduction

This chapter could have been placed at the beginning of the volume because the points raised are central to the application of cryopreservation. We chose to place it at the end, however, as a summary, and to provide a connection to the future and to those who will thaw and thereby inherit what we cryopreserve.

During the last half of the twentieth century, we have witnessed a series of remarkable technologic advances in the exploration of space; a highlight of that exploration was the Apollo moon landing of July 1969. Now, as our unmanned probes rove the surfaces of distant planets, we contemplate the completion of space stations under construction and the personal exploration of Mars and the more distant planets. Yet for all our enthusiasm, we are bound essentially to our own solar system, held back by the vast reaches between universes and the limitations of speed and energy. Even if we could attain the speed of light, a round trip to our nearest neighbor, the star Alpha Centauri, would take almost 9 yr.

We have witnessed, too, a series of remarkable technical advances in the physical sciences, especially genetics. We have deciphered the genetic code and developed the means for gene splicing and cloning. We have transferred genes from one organism to another and in so-doing we have created novel life forms. But unlike the situation with space travel, genetic exploration is virtually without limit. We are developing the techniques for gene therapy, for the repair or replacement of mutant genes, and even as these words are written, the Human Genome Project is underway -- a massive and comprehensive approach to the determination of the entire genetic sequence of a human being. Although the project may take several yr, there is no reason to doubt that it will be completed (author update: the initial working draft sequence of the human genome was published in 2001). Indeed some 25 prokaryotic genomes have already been sequenced (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov).

Watchel, S. S. and T. R. Tiersch. 2011. Ethical Considerations in the Application of Cryopreservation to Aquatic Species. *In: Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 984-994.

As for the genetics of reproduction, we have learned how to make infertile couples fertile. We can treat women such that they produce many eggs in a single ovulatory cycle. We can use the eggs to produce embryos in test tubes. We can cut the embryos in half to make twins, we can fuse two to make one, we can clone one to make four, we can freeze the embryos and store them and we can predetermine their sex. We can transfer them into surrogate mothers who will carry them to birth.

Given the rate of scientific discovery, it would seem wise to consider standards of scientific behavior, for there is little consensus concerning the legitimacy or the morality of our novel technologies, and we are manipulating the very elements of the generative process. Even the idea that Man cannot make life, that life is uniquely a creation of the Divine, can now reasonably be challenged. Yet the available and other, developing, techniques could be used to protect and conserve any of several endangered species, mammalian and non-mammalian. When particular environments are threatened, for example, germ cells and embryos of the species at risk -- even intact individuals -- might be cryopreserved, with a view to the thawing and "seeding" of favorable environments in the future. But these novel methods raise novel questions -- the question of *multigenerational inbreeding*, for instance, the inadvertent mating of individual animals with their descendants, the question of *temporal exotics*, species out of synchrony with their environment because of changes occurring during their period of cryopreservation, and so forth.

In this chapter, we discuss reproductive methods that have been developed in mammals and their potential application to conservation of the non-mammalian species -- fishes in particular. We conclude with a consideration of some of the ethical and religious questions germane to the application of these methods.

Assisted Reproductive Technologies (ART)

Assisted Reproductive Technologies refers to any of several methods used to enhance the reproductive potential of individuals and includes such techniques as *in vitro* fertilization (IVF), cryopreservation, twinning and cloning, embryo transfer, and surrogate motherhood, and, in the human and domestic mammals, pre-implantation and prenatal diagnosis and sex selection. Many of these methods were developed in laboratory rodents and in the larger domestic species. Pregnancy resulting from embryo transfer in cattle was first reported in 1951 (Seidel 1981); mice born from frozen embryos were first described in 1972, and cattle produced from frozen embryos were first reported in 1973 (Hafez 1992).

Children born as a result of IVF are called "test-tube babies." The first such was Louise Brown, born in England in 1978. Nearly 15,000 test tube babies were born in the United States and Canada in 1994 and 1995, the last 2 yr for which figures are available (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine 1994, 1998) and over 300,000 children have been produced by IVF Worldwide (Institute for Science, Law, and Technology Working Group 1998). Following hormonal stimulation in women undergoing IVF, as many as 20 (or more) embryos can be produced, but only a few are transferred. The rest are often cryopreserved.

The first child resulting from transfer of a cryopreserved embryo was born in 1984. Since then the technique has been widely used. More than 7,000 frozen human embryos were thawed and transferred in the United States and Canada in 1994 and again in 1995. At present there are

tens, perhaps hundreds of thousands of human embryos frozen in liquid nitrogen (LN_2), awaiting transfer or other disposition.

Cryopreservation of human embryos has generated extraordinary circustances that few would have contemplated even in the recent past. An example involves two embryos frozen in Australia after being produced by IVF with sperm from an anonymous donor. The parents-to-be were killed in an air crash and the question arose whether the embryos should be thawed and transferred into a surrogate mother. Should those embryos be given the chance for survival? And if they survived should they inherit their parents' estate, valued at seven million dollars? A committee was formed to provide guidelines. The committee ruled that couples enrolled in IVF programs should make provisions concerning the disposition of frozen embryos, that in the absence of such provisions, embryos should be destroyed. But the Victoria Parliament ruled that all embryos should have the chance to be transferred and born, even in the absence of provisions (Davis 1990).

Conservation by ART in the Non-human Mammals

As implied above, cryopreservation and other technologies raise fundamental issues about the legal status of, and our moral obligations to, the developing human, but they also provide the opportunity to rescue species whose numbers have been critically depleted by deforestation, pollution or overharvest. Cryopreservation in particular would enable storage and safeguarding of germ cells and embryos for development at a time and place favorable to the survival and well-being of the species. Once thawed, embryos could be cloned (Willadsen 1986). Given a favorable environment, the combination of cryopreservation and cloning could enable rapid re-population.

Cryopreservation is straightforward. The embryos or germ cells are placed in a cryoprotectant such as glycerol to protect against the formation of lethal ice crystals, and the temperature of the solution is reduced gradually, often in a programmable cooling machine. Crystallization of the medium is induced and the embryos are plunged into LN_2 for extended storage. For thawing, the embryos are merely removed from the LN_2 and allowed to warm quickly in air.

This or similar techniques could work well with fish and other taxa in which embryos develop in the open environment, but it may be more difficult to implement rescue of mammalian species such as the cheetah, in which uterine gestation is a necessary component of development. This difficulty could be circumvented by use of surrogate mothers from related species but this is a development for the future. Where germ cells are available instead of embryos, the embryos could be produced by IVF. This would enable preimplantation diagnosis of the embryos, and thereby selection of gender and other desired traits.

The Ethics of ART: Religious and Secular Considerations

Considerable discussion has been generated by the development and application of ART in humans and domestic mammals. The recent cloning of a sheep was widely reported in the press and electronic media, for example, and as might be expected, it created a stir in certain religious circles. In a news article in Nature (Masood 1997), Jeremy Rifkin, President of the Foundation on Economic Trends in Washington was described as heading a "coalition of 300 religious and ethics organizations" calling for a worldwide ban on human cloning and suggesting

that human cloning should carry a penalty "on a par with rape, child abuse and murder." Yet the cloning of human embryos had already been performed 4 yr earlier (Hall et al. 1993).

The reaction to the applications of ART in conservation of the domestic species would be more subdued, but the questions raised are no less compelling. Is there really a need for species preservation? How are the effort and expense of preservation justified? Are we morally obliged to preserve endangered species? Which species should be selected for rescue and which should not? Should techniques such as cloning be banned?

In the book, *Principles of Conservation Biology*, Callicott (1994) identifies "four categories of the instructional value of biodiversity:" 1) goods, such as foods, fuels, materials for clothing, drugs; 2) services, such as pollination, nitrogen fixation, etc.; 3) information, such as that concerned with genetic engineering and pure and applied science, and 4) the psycho-spiritual, such as that concerned with aesthetic beauty and religious awe. The author notes that the drug, vincristine, obtained from the Madagascar periwinkle, is used in the standard treatment of childhood leukemia, and that the plant grows in an area which is threatened with "wholesale destruction." It is asked whether other yet undiscovered species could provide additional benefits, and whether these potentially valuable species are being destroyed routinely by careless exploitation and destruction of habitat.

In considering the potential loss of information the author compares what he refers to as "mindless destruction of biodiversity" with book burning. From the perspective of aesthetics and religion, he compares it with "vandalizing an art gallery or desecrating a church." Later the author points out that there are religious precedents or conservation and in particular for the protection of endangered species. Indeed the Bible and commentaries are full of references implying the stewardship of Man over the animals, and his responsibility to protect them:

Rav Judah said in the name of Rav: a man is forbidden to eat before he gives food to his beast, because it says (Deuteronomy 11:15) 'and I will give grass in your fields for your cattle' and then 'you will eat and be satisfied.'

Babylonian Talmud: Berachot 40a

In it (the Sabbath day) you shall not do any manner of work, you nor your son nor your daughter, not your man-servant nor your maid-servant, nor your ox, nor your ass, nor any of your beasts...

Deuteronomy 5:14; see also Exodus 20:10

In this connction, it is interesting that the forms of work that are otherwise forbidden on the Sabbath (unloading for example) must be done to reduce animal suffering (Babylonian Talmud: Shabbat 128b). Thus, in imitation of the Divine, Man is cast in the role of the caretaker obliged to protect the various species in his stewardship. Given the novel technologies discussed above, this would imply an obligation to apply those and other developing technologies to the protection of all species, and in particular endangered species.

Is there guidance on how to proceed in this obligation? Perhaps an example comes from the response of the Buddha to a monk seeking answers to the questions of higher philosophy.

"*A man was shot by a poisoned arrow. With the arrowhead still embedded within him, his relatives raced to find a doctor. As the doctor was preparing to cut out the arrow-head, the man said, "Wait! I will not let you take out this arrowhead until you tell me the name of the man who shot me. Where did he live? What caste was he? What kind of arrow did he use? Did he use*

a bow or a cross-bow? What was the arrow made of? Of what was the bow made? Of what was the bow-string made? What kind of feather was attached to the end of the arrow? Until I find out the answers to these questions, I will not let you take this arrow out."

The Buddha was teaching that it is important to relieve human suffering and to avoid wasting time in vain pursuits and debates. In this light, do we need to know all of the details associated with cryopreservation of sperm of endangered species before we engage in the work? How do we decide that we know enough?

Conservation by Cryopreservation in Aquatic Species

While many areas of human bioethics are well codified, and others are still being framed, the ethics of cryopreservation of gametes and embryos of aquatic species are unexplored. Although certain questions can be addressed by looking to established areas such as conservation ethics (Callicott 1994), ethical concerns for cryopreservation pose unique problems. In this section we address several examples of ethical questions involving application of cryopreservation to threatened and endangered (T & E) species of fishes. This is relevant because fully one third of all fish species are now threatened with extinction (Tuxill and Bright 1998). These questions relate to ethical problems in other arenas as well. The four primary examples are: 1) use and disposal of samples; 2) multigenerational inbreeding; 3) temporal exotic species, and 4) eco-temporal effects.

Use and Disposal of Samples

Cryopreservation is actually a form of time travel, because frozen samples will likely remain viable well beyond our lifetimes. The social, biological, and political contexts that exist when we freeze samples will most probably be irrelevant given passage of enough time before thawing. Thus we are freezing samples for potentially unknown uses in the future.

Samples frozen today for research such as optimization of cryoprotectants or cooling rates may eventually need to be used for breeding purposes. Or, samples frozen for use in breeding may only be minimally viable due to use of unrefined techniques. In this first case, we may not be able to gain maximum benefit for breeding purposes from the sample because insufficient biological information on the genetic characteristics of the donor fish was recorded (the samples were not intended for breeding use). Should we dispose of the samples to ensure genetic integrity of populations? In the sec case, we have poor albeit well-documented samples occupying potentially limited space that could be occupied by more valuable (viable) samples. Even if space were not limiting, potentially fertilizable eggs could be lost by use of inferior sperm. Should we keep the samples for some other use (e. g. genetic analysis) or should we dispose of them?

In each of these cases we must weigh the costs and benefits of using thawed samples for purposes different than those at the time of freezing. Neither case is unlikely -- almost certainly each will occur repeatedly in the future. The ethical dilemma would also be intensified given the survival status of the species. Flawed samples become more valuable based on simple supply and demand. It should be noted that the market and non-market values have yet to be established for such samples. A new economic discipline, environmental or ecological economics, is emerging to deal with issues in valuation of natural resources (e.g. Costanza et al. 1997). At present, the

methodologies are controversial (Pimm 1997) and have not been applied to cryopreservation in aquatic species.

Are there solutions? Perhaps systematic record-keeping and a centralized data base should accompany any sample cryopreserved for any reason (though costly today, this may prove to be a bargain in the future). Perhaps analysis of DNA from a sample of frozen material could resolve problems associated with genetic husbandry. The problems of poor quality samples and poor quality control are more difficult. Perhaps technology such as microinjection of sperm nuclei or intracytoplasmic sperm injection could make even poor samples useful.

Multigenerational Inbreeding

This is another question of usage. When working with T & E species it is often difficult to obtain samples in a uniform and systematic fashion. Fish may congregate only in specific areas during spawning season (e. g. over gravel substrates) and only certain males may yield copious quantities of high quality semen. In short, a few males from a few populations may provide disproportionate excesses of the total volume of sperm cryopreserved. This is not unlikely given that the doctrine of "it's all we have so we better freeze it" seems quite reasonable when faced with the imminent collapse of a species.

Let us consider use of these samples. With frequent thawing and breeding over time, it is possible to create a situation wherein sperm from a single over-represented male are used to fertilize eggs produced by his offspring for successive generations -- multigenerational inbreeding by overuse of a single sire (also an extreme version of recurrent founder effect). This is, like our first examples, not an unlikely event, or one without precedent. The overuse of cryopreserved sperm of a single Holstein bull (named "Elevation"). His sperm produced more than 2 million offspring. Of course, while inbreeding in domesticated animals may be desirable to some extent to fix desired traits, it is not desirable for natural populations of T & E species.

Is there a solution to this problem? Yes, but not without ethical considerations. Limits could be set *a priori* on the volume of sperm frozen from any one male regardless of the population level of a species. There is another relevant concept here. Frozen samples could be viewed as a means to reconstitute genetic variation in the form of fish. The frozen lifetime of sperm could be set (again *a priori*) and the sons produced from cryopreserved sperm could be used to provide sperm to be frozen for future reconstitution. Thus frozen sperm from any one male would be carried forward for only a generation or two. Even inferior sperm samples could be used to produce a few males in this scheme (the females would be useful too, of course), which would then provide sperm for production of fishes and for cryopreservation using newer improved techniques.

Temporal Exotic Species

Our first two ethical questions have dealt with problems essentially of inventory and use, which is the domain of fish production. Our next two questions address what happens to the fish that are produced from cryopreserved material. For these questions we can focus on application of a hypothetical method for cryopreservation of fish embryos (to be developed in the future).

The concept and consequences of exotic species are familiar in fisheries, and this topic was the subject of a book recently published by the American Fisheries Society (Schramm and Piper 1995). When a species is introduced outside its natural range it can cause significant alteration in the new habitat. Often this is detrimental to the resident organisms. Inasmuch as cryopreservation is a form of time travel, another type of exotic species is therefore possible: the temporal exotic -- a species re-established in its natural range some time subsequent to its

extirpation from that range. The degree of ecological displacement of this species would be related to the degree of temporal displacement. As an extreme example, what if the extinct placoderm fish, *Dunkleosteus* (Romer 1966), were re-established in its former range? This 10-m long predator (extinct for some 350 million yr) presumably capable of shearing telephone poles in its jaw plates could have dramatic effects on its reclaimed territories (Figure 1). Though not as dramatic, the re-establishment of an extirpated large predator could affect waterways in the future. The Colorado squawfish *Ptychocheilus lucius* which attains sizes of 2 m and 100 Kg is currently listed as endangered and would be a candidate for reintroduction in the Colorado River system in the future.

Other examples of temporal exotics could include commensal organisms such as innocuous bacterial flora, that when reintroduced, say 50 yr after extirpation, could assume new roles as pathogens. The inadvertent transfer of today's pathogens to the future could also be dramatic. We need only look at clinical examples of human disease provided by the reemgergence of tuberculosis and polio after immunization programs were curtailed. Thus we must consider the possibility, if not the likelihood, that the organisms and germ cells we freeze today can have detrimental effects in the future, even if the organisms and germ cells are innocuous in the present.

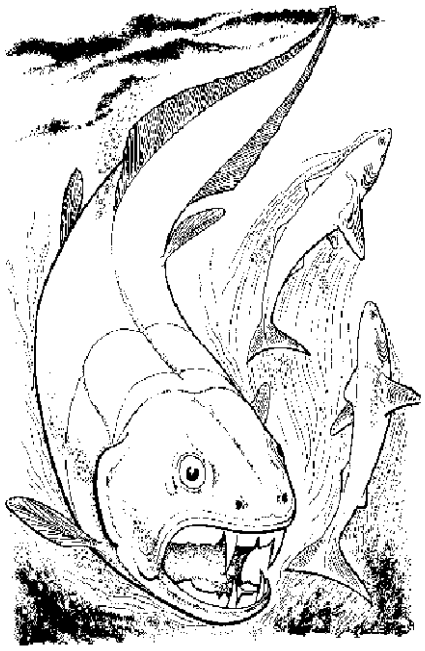

Figure 1. The Devonian predator *Dunkleosteus* depicted menacing primitive sharks. Re-establishment of this 10-meter long extinct fish into formerly occupied territories would be an extreme example of a temporal exotic species. Reproduced with permission of Spizzirri Publishing Company from "*An Educational Coloring Book of Prehistoric Fish*."

Eco-temporal Effects

All of the preceding examples have addressed implicitly the "emergency response mode" that is currently operational with T & E species. Given the precipitous declines now occurring, we often provide attention to the most endangered of species first and presume that anything done is better than doing nothing. Unfortunately there is often little time to plan, for some species such as the humpback chub *Gila cypha* have only a few hundred representatives left in the wild. This has led to an "act first, think later" approach. The result is a collection of single-species programs, each addressing a dying population. However, species seldom go extinct by themselves. In reality it is specific habitats and communities that are endangered and become extinct. In view of this precept of conservation biology, is it reasonable to preserve embryos of a particular fish and not to preserve embryos of other members of the aquatic community? It is beneficial to thaw fish embryos 50 yr from now for introduction into suitable habitat if the food sources (for example) are gone? Should we thus cryopreserve insects, plants, microbes and other prominent members of aquatic systems such as "keystone species" (New 1995) to ensure the future success of embryos frozen today? This is a particularly thorny ethical question.

The four preceding questions were used to introduce a range of ethical problems related to the use of cryopreservation with aquatic species. Clearly there are other ethical issues. For

example, how will resources be allocated? Do we need to employ a triage system? Should we cryopreserve only those species that cannot be saved by present-d management practices (the same approach as freezing the bodies of people with diseases that are incurable today)?

There are further questions raised related to the social impacts of cryopreservation. Does cryopreservation lead to decreased protection of sensitive habitats? It has been argued that frozen sperm can open the path for future development and habitat destruction by reducing the perception that species are actually imperiled. It could also be argued that freezing of sperm or embryos is a legitimate form of mitigation to compensate for destruction of habitat (we can build a new dam if we "protect" the displaced species by cryopreservation). Precautions for the use of cryopreservation with T & E species need to be discussed (Table 1).

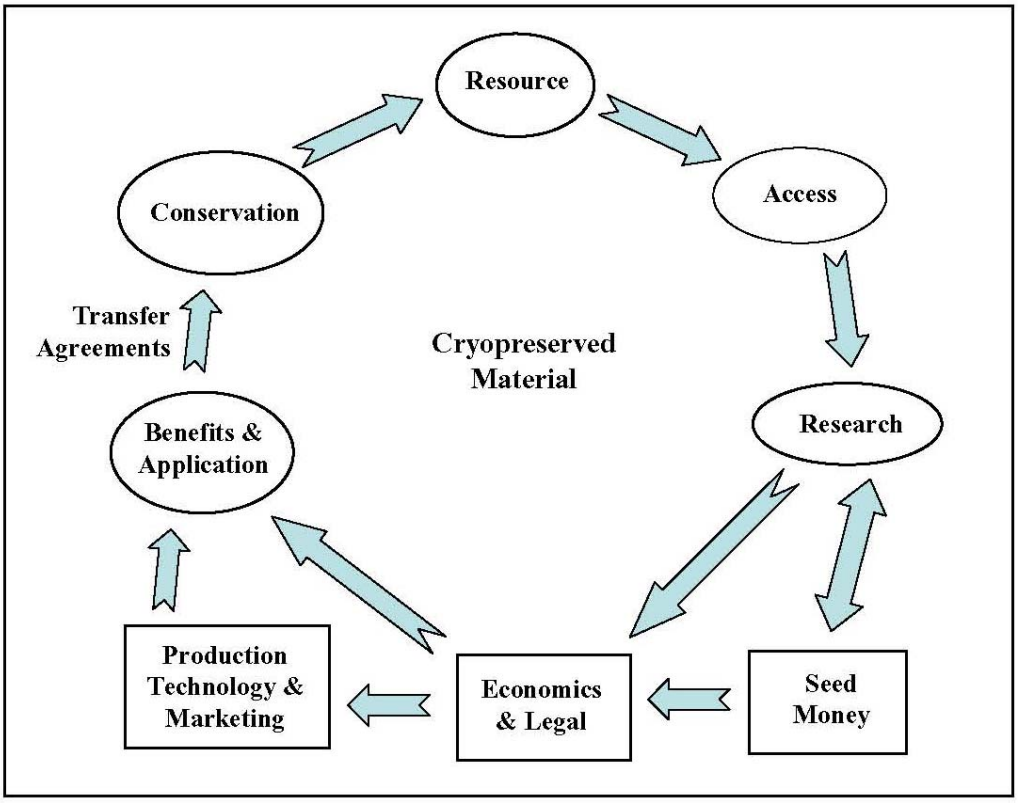

Figure 2. A scheme for utilizing market value of natural resources to generate funding for protection of the resource. This scheme would involve commercialization by the private sector (in rectangles) of public resources (ovals) and would involve resolution of legal, economic, social, biological, and ethical questions before introduction.

Other questions pertain to the market value of the sperm of an endangered species or the frozen embryos of an extinct species. Given that value is related to supply and demand, what is the dollar value of the last remaining embryos on Earth of a particular extinct species? Would cryopreservation of T & E species be best served by private sector involvement? It could be argued that private businesses (with government regulation) are capable of development and management of germplasm or embryo repositories for T & E species. This leads to the concept of resource privatization (Figure 2), where the market value of natural resources can in part be used to protect the resources (Vogel 1994, Macilwain 1998).

Table 1. Caveats for application of cryopreservation to threatened and endangered species.

1)	Technology is seductive and not always necessary.
2)	The techniques of cryopreservation are unrefined or non-existent for most species.
3)	Cryopreservation is a tool, not a final solution.
4)	Cryopreservation does not protect habitat.
5)	Cryopreservation does not replace existing management plans.
6)	Cryopreservation can buy time or waste resources.
7)	There are few ethical guidelines for the application of cryopreservation to species conservation.

As a final example of potential questions, we should consider the extent to which technology should be applied. Consider the use of androgenesis. This is a process (with natural examples) allowing production of fish with nuclear DNA originating from sperm only. When used as a technique for genetic manipulation, fish can be produced from cryopreserved sperm and irradiated eggs (in which the nuclear DNA has been destroyed). This has been demonstrated in rainbow trout *Oncorhynchus mykiss* for example (Thorgard et al., this volume). In a worst-case scenario, cryopreserved sperm from an extinct species could be used to provide the nuclear DNA for use with eggs from a closely related "donor species." The resulting fish would combine nuclear and mitochondrial (cytoplasmic) inheritance from two species. Is this a suitable substitute for the original extinct species?

Table 2. Points for discussion of ethical considerations for the application of cryopreservation to aquatic species.

1)	Cryopreservation is a form of time travel.
2)	We cannot be certain of how frozen samples will be used in the future.
3)	Use and disposal of cryopreserved samples will require rules.
4)	Quality control and quality assurance will be required.
5)	The value of frozen samples is determined by record-keeping and database management.
6)	We should consider the use of cryopreserved material for genetic reconstitution instead of production.
7)	Genetic management can be intentional or unintentional.
8)	Organisms considered to be desirable or innocuous today, may not be so in the future.
9)	Diseases of today could be worse when reintroduced in the future.
10)	We cannot predict the interactions of today's organisms with those of the future.
11)	Species do not live in isolation: it takes a community.
12)	Resource allocation is necessary for cryopreservation.
13)	Cryopreservation could be viewed as mitigation for development activity.
14)	Private sector involvement is possible for cryopreservation of T & E species.
15)	Saving a species can transform it.

Clearly the ethical considerations are numerous and important (Table 2). At present, we not only lack good answers, we also lack good questions. In order to give relevance to our actions today, we are obliged to develop an appreciation for the scope and nature of the problems of the future. Application of cryopreservation to aquatic species, especially T & E species, is an unexplored realm of bioethics that will increase in importance with time. Perhaps by asking the question "What will they be thinking 50 yr from now?" We can avoid having those in the future ask "Why didn't they think about this 50 yr ago?"

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Annotated Bibliography of Developments in the Last Decade

Reproductive ethics has been a popular and necessary topic since the inception of assisted reproductive technologies. Most thought has focused on questions relevant to humans and with techniques related to embryo curation and artificial insemination. Recent reviews have attempted to summarize a comprehensive view of ethical considerations relating to gametes. Few publications, however, mention considerations relating to germplasm cryopreservation in aquatic species. *Development of guiding principles in this area will become increasingly important as cryopreservation continues to develop as a research field and area of commercial interest.*

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New Chapter

Development of High-throughput Cryopreservation for Aquatic Species

E Hu and Terrence R. Tiersch

Introduction

High-throughput cryopreservation has been widely applied in livestock industries such as dairy for decades (Pickett and Berndtson 1974), and cryopreserved germplasm constitutes an independent industry encompassing animal breeding, preservation of genetic diversity, and medical research. Fish sperm cryopreservation was demonstrated at about the same time as for humans and livestock species (Blaxter 1953), however, despite the large-scale application in mammals, it remains at a research scale for aquatic species. During the past decades, there have been more than 200 aquatic species reported for sperm cryopreservation (Tiersch and Mazik 2000) and this number is increasing (although an accurate current estimate is not available). However, there is currently no system for large-scale production of cryopreserved germplasm of aquatic species. Recently, a survey of fish culturists revealed a high demand for genetic improvements of the type that can be provided by cryopreservation (Boever 2006). Therefore, high-throughput cryopreservation for aquatic species comes with high expectations.

As stated above, high-throughput cryopreservation has been widely applied in livestock industries for decades. Its development grew out of advances made in the laboratory, and was scaled up for increased processing speed, capability for mass production, and quality assurance. Available equipment and processes in place for livestock and biomedical applications have been tested for feasibility of use with aquatic species (e.g., Haffray et al. 2008), with extensive work for more than 15 yr at the T. E. Patrick Dairy Improvement Center of the Louisiana State University Agricultural Center. For example, sperm of blue catfish *Ictalurus furcatus* was cryopreserved at this commercial facility using procedures developed for dairy bulls (Lang et al. 2003), as was done for sperm of common carp *Cyprinus carpio*, channel catfish *Ictalurus punctatus*, three species of sturgeon (*Scaphirhynchus* and *Acipenser*), striped bass *Morone saxitilis*, white bass *M. chrysops*, yellow bass *M. mississippiensis*, and marine species such as spotted seatrout *Cynoscion nebulosus*, red snapper *Lutjanus campechanus* (Roppolo 2000), and diploid and tetraploid Pacific oysters *Crassostrea gigas* (Dong et al. 2005, 2007a). Since then protocols and automated equipment developed for mammals have been specifically adapted for use with aquatic species (described below) including zebrafish *Danio rerio* (Yang et al. 2007), blue catfish (Hu et al. 2010), Eastern oyster *Crassostrea virginica* (Yang et al. unpublished data), channel catfish, and Atlantic salmon *Salmo salar* (Hu et al. unpublished data).

Automated Equipment

High-throughput can be achieved in different ways, and adoption of automated systems is one of the most efficient methods. An automated system named MAPI (CryoBioSystem Inc. Paris, France) for loading, sealing, labeling and reading of straws has been developed for mammalian high-throughput. Adaptation of this equipment has been evaluated for aquatic species to enable commercial-scale use of cryopreserved sperm (e.g., Hu et al. 2010). Briefly, upon mixing with cryoprotectant, sperm samples are placed on the MAPI system, and filling,

Hu, E and T. R. Tiersch. 2011. Development of High-throughput Cryopreservation for Aquatic Species. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T. R. Tiersch and C. C. Green, editors. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 995-1003.

sealing and labeling of the straws are controlled by a proprietary computer program (SIDE, CryoBioSystem, Inc.). Samples are drawn into plastic 0.5-mL (CBS) straws by vacuum, and are continuously transferred to the sealing platform where both ends are sealed by use of 158 °C heat clamps. The straws are labeled with alphanumeric information and bar-coding on the identification jacket with an ink printer (A400, Domino, IL, USA) before transfer for label verification and quality control evaluation. This system can routinely produce 11 straws per min.

Freezing follows using standardized procedures. Straws are arrayed on horizontal racks (40 per rack) and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV, France) with a capacity of 280 straws per freezing cycle. If the thermal mass is not controlled at each freezing (e.g. by adding "dummy" straws), the number of straws is regulated within a tight range. The cooling program is initiated at 15-30 min after addition of cryoprotectant. The cooling rate can be programmed from 1 °C /min to 40 °C/min (based on chamber temperature) and various rates are studied (detailed below). When the final target temperature (-80 °C) is reached and held for 5 min, the samples are removed and sorted under liquid nitrogen into 12-compartment storage containers (Daisy goblets, reference number: 015144, Cryo Bio System) for long-term storage in liquid nitrogen.

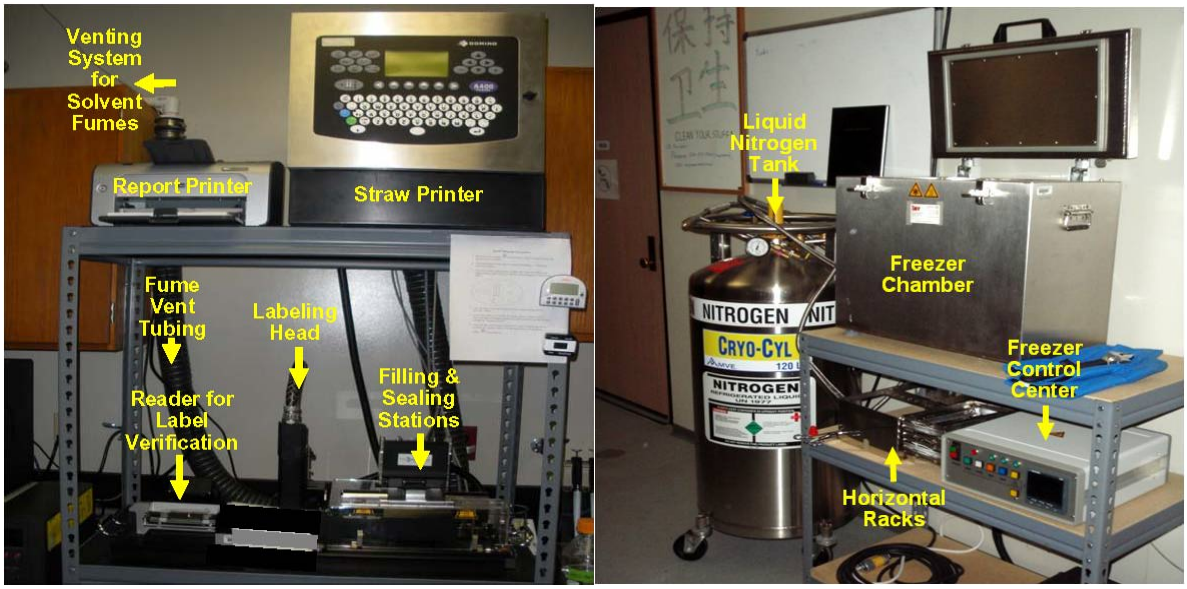

Figure 1. In the MAPI system (left), sperm suspensions are loaded and packaged into straws at filling and sealing stations, labeled under the labeling head driven by the straw printer, and pass a barcode reader for label verification. Next, the straws are laid on horizontal racks and placed in a programmable freezer (right) controlled by regulating liquid nitrogen flow from a pressurized storage tank. To see MAPI in action, *click here*.

High-throughput Pathways

Critical aspects of high throughput are arranging the sequence of procedures and balancing the inputs and outputs between connected steps. For any sequence developed, industrial designers and economists can further evaluate production resource utility and processing costs. In general for fish that require dissection for sperm collection, we have identified 5 major steps: dissection, sample processing, freezing and sorting, storage, and

utilization (Figure 2). An industrial engineering approach would focus on reducing the time and cost at each step to increase the efficiency of the production cycle, and reduce the possibility of bottlenecks between steps. In preliminary economic analyses of blue catfish, the estimated cost per straw was US$1.50 without labor, and each month there was an additive storage cost of US$0.02 per straw for supplies such as liquid nitrogen (our unpublished data).

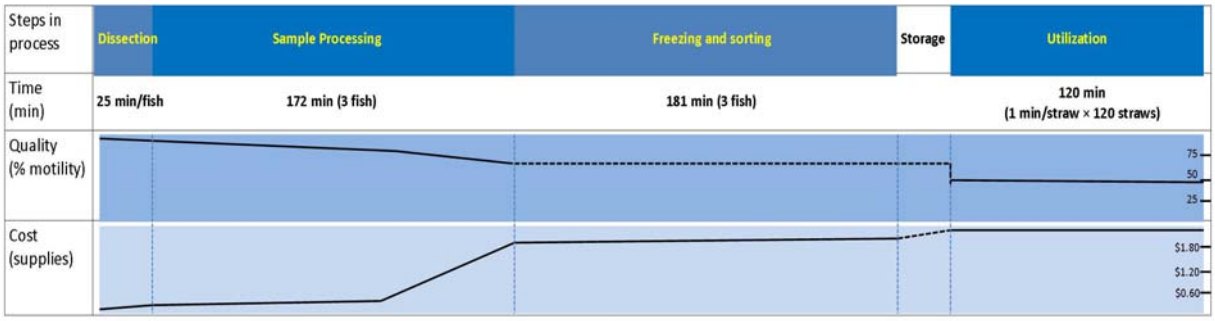

Figure 2. A simplified (5-step) schematic of the cryopreservation process for blue catfish. Each step is displayed horizontally in proportion to the amount of time required (in min) in relation to the sperm quality observed (expressed as percent motility) and supply costs (US$) for the number of fish or straws handled at each step. The long time intervals in the sample processing, freezing and sorting, and utilization steps were recognized as bottlenecks to be addressed in future research.

Commercial-scale Evaluation of High-throughput Processing

High-throughput cryopreservation is only beginning in aquatic species. Currently, marine species such as southern flounder *Paralichthys lethostigma* and Pacific oyster have been tested with laboratory-scale fertilization, anadromous species such as Atlantic salmon have been tested with hatchery-scale fertilization, and blue catfish was tested at a commercial scale with more than 1,400 straws in a single trial with 242 channel catfish females (Table 1). With a specified sperm-to-egg ratio (5×10^5 sperm per egg), thawed sperm had the same fertilization capability as fresh sperm on a per-male basis, and thawed sperm yielded production of 200,000 fry per day.

Table 1. Commercial-scale production parameters for use of high-throughput cryopreservation of blue catfish sperm. Cryopreseved sperm from 17 males and fresh sperm from 16 males were cross-tested during 4 d using eggs from 242 females. (P-values obtained from T-test).

Parameter	Thawed sperm	Fresh sperm	P-value
Sperm use per day (cells)	$17 \pm 6 \times 10^{10}$	$24 \pm 12 \times 10^{10}$	0.173
Hatching rate	43 ± 15%	52 ± 4%	0.158
Sacfry produced (per day)	$17 \pm 7 \times 10^4$	$28 \pm 7 \times 10^4$	0.034

Development of quality control and assurance are as important as development of proper protocols, and are likewise only just beginning for high throughput application in aquatic species. Male-to-male variation in the quality of cryopreserved sperm is commonly observed in species including mammals, fishes, and invertebrates (Mazur et al. 2008, Yang et al. 2009), although the underlying mechanisms for the variability remain unresolved (Dong et al. 2007b,

Tiersch et al. 2007). Unlike comparable previous reports in aquatic species, no significant variation was observed in post-thaw motility and fertility among ten blue catfish males (Figure 3) used in our commercial-scale study (Hu et al. 2011). This is desirable for high-throughput technology because it can provide products with consistent and predictable output. Possible explanations for the lack of variability include: 1) a small sample size of 10 males (although comparable to previous studies); 2) unknown attributes of the reproductive biology of blue catfish; 3) tight standardization of sperm concentration which limited variation in response to toxicity or cryopreservation, and 4) the setting of threshold of initial motilities (>40%) at the beginning of the process which reduced the variability of fresh sperm quality (comparable to previous studies). Although these factors alone or in combination could explain the low observed variability, we propose that the use of a tightly standardized protocol, especially for sperm concentration (given that the majority of previous studies in aquatic species did not control this critical variable), would be consistent with production of a predictable and consistent range of post-thaw quality.

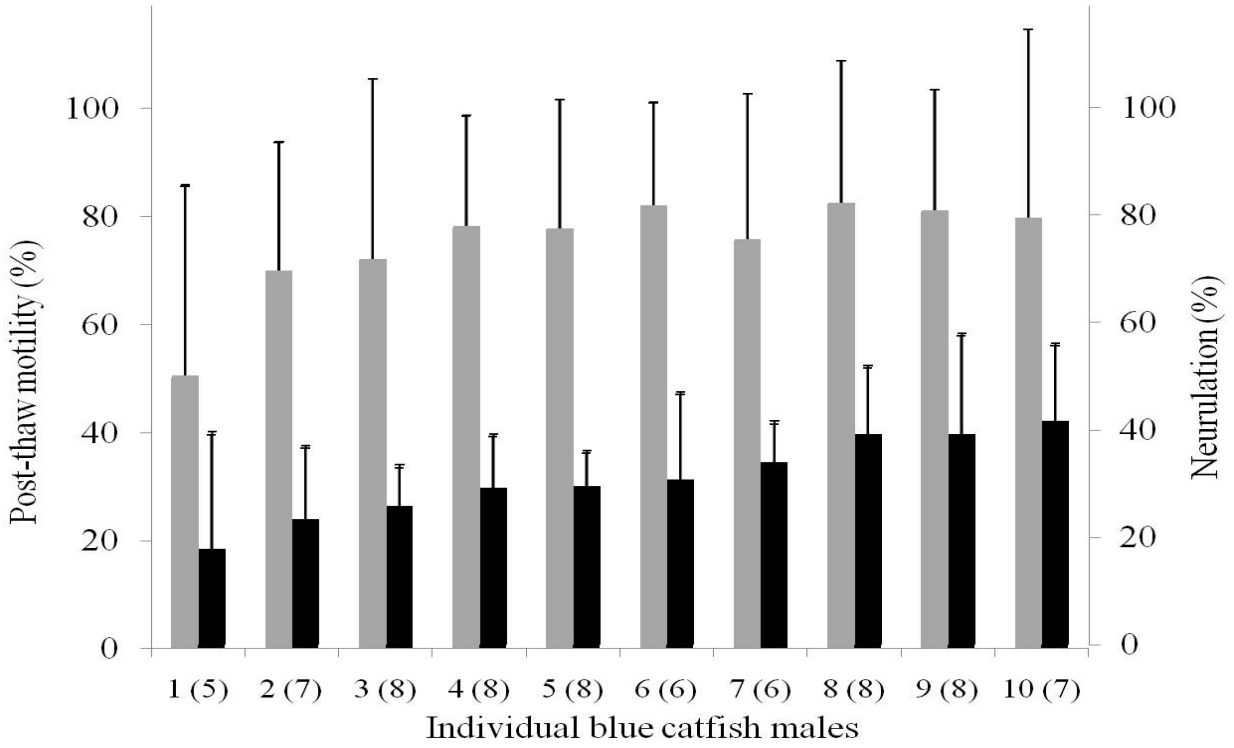

Figure 3. Sperm from 10 blue catfish males was cryopreserved using 1×10^9 cells/ml, 10% methanol, and a 5 °C/min cooling rate. Sperm were thawed and used to fertilize eggs from 5-8 channel catfish females (numbers of females indicated in parentheses). Post-thaw motility (dark bars) and neurulation (light bars) were used to evaluate male-to-male variation. Post-thaw motility and fertility of thawed sperm were not significantly different among the 10 individuals (Hu et al. 2011).

High levels of reliability are necessary for any technology to become commercially relevant. For high-throughput cryopreservation of aquatic sperm to move from the laboratory into commercial application, the process can no longer be viewed as simple combinations of cryobiological factors. Instead, from a manufacturing point of view, the process needs to be recognized as a sequence of operations relying on different equipment and resources (Figure 4).

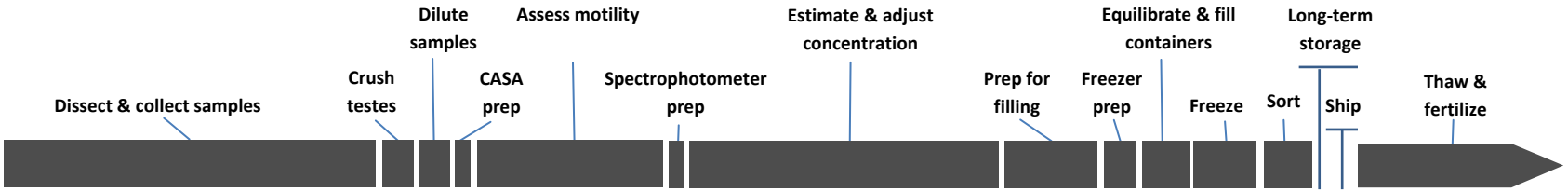

Figure 4. Schematic representation of a cryopreservation pathway established for sperm collection by dissection of the testis (e.g., for blue catfish or zebrafish *Danio rerio*). In its basic form, the process comprises 15 standardized, integrated steps from sample collection through thawing and use for fertilization. The steps are presented as rectangles sized in proportion to the total time required for processing and cryopreservation of samples from 20 individual males. CASA: computer-assisted sperm analysis.

With this perspective, further studies can focus on the optimization of overall system efficiency, while balancing biological and other factors, leading to a robust commercial model.

Thus, establishment of process pathways will assist in ensuring efficiency and quality of high-throughput products. In addition, these process pathways can be standardized or harmonized to provide central pathways for movement of material into comprehensive germplasm repositories achieving high throughput in two ways: intrinsically by themselves, and extrinsically by acting as a funnel for supplemental sources of cryopreserved material (Figure 5). New equipment, supplies, and reagents will eventually be necessary to

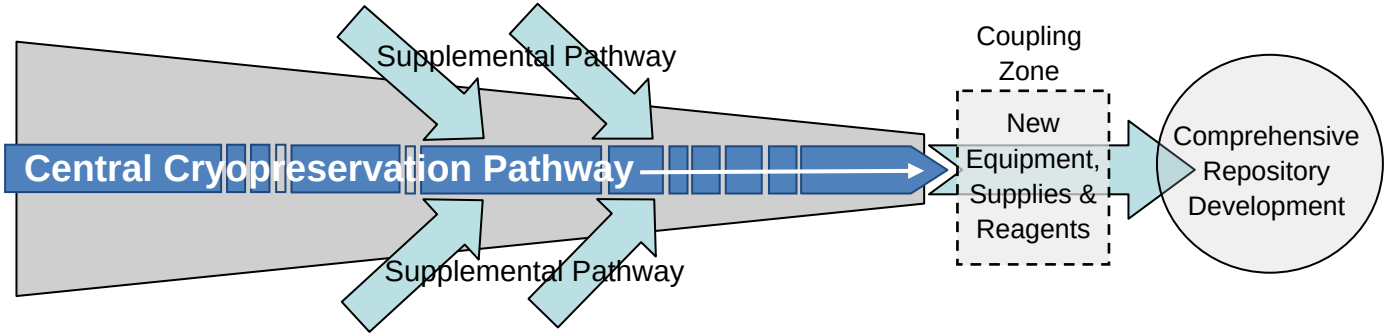

Figure 5. Conceptual approach for reinforcing the existing cryopreservation pathway and using it as a central route to funnel materials from other laboratories through a coupling zone that will integrate future commercial advances in high-throughput cryopreservation technology specific for aquatic species to support development of large repositories capable of handling hundreds of thousands of samples.

fully address the specific needs of aquatic species, although such a system could begin operation by adaptation of commercially available products developed for livestock and biomedical application.

Future Needs for Commercial Application of Cryopreservation in Aquatic Species

For high-throughput cryopreservation to assume a functioning role in assisting aquaculture production and aquatic species conservation it has to proceed beyond development of the initial technical requirements and requires changes in conventional thinking. For example, high throughput by definition involves large numbers of samples. Inherent in this is the need to minimize waste and inefficiency because small mistakes or inefficiencies are multiplied and can become costly. For example, use of a sperm concentration of 10^8 cells/mL was not biologically different from the use of 10^9 cells/mL (Hu et al. 2011), and at a typical laboratory scale of 100 straws or fewer, not economically different as well. However, in a high-throughput setting for the same total number of sperm cells, the use of 10^8 cells/mL would require 20,000 straws compared to only 2,000 straws for 10^9 cells/mL – a difference of US$27,000 (US$30,000 vs. US$3,000) for the numbers of straws used in our commercial blue catfish trial.

A series of activities are required to be in place for full-scale application. These activities have not yet been fully implemented anywhere for aquatic species. There are problems and barriers at each step, but getting started is usually the hardest part. The entry-level requirements for equipment, facilities, and training are high and force potential users to focus on technology development and technical problems (e.g., Caffey and Tiersch 2000a,b). However after these hurdles are passed, the focus can shift to coordination of activities and realization of the substantial opportunities provided by cryopreservation. This would include establishment of high-throughput capabilities, which has recently been recognized as a focus for necessary research by the US National Institutes of Health for biomedical models including fishes (workshop summary available at: *www.ncrr.nih.gov/publications/#reports*). A final phase of maturation in application would see cooperation and connections forming among governmental agencies, non-governmental organizations, academic institutions, and private companies. The major activities in this potential pathway for application would include (Tiersch 2008):

1) Development of necessary technical capabilities and facilities at well-funded and secure locations sited near aquatic species activities, or easily accessed by overnight shipping services.
2) Establishment of training programs for procedural efficiency, and education of personnel.
3) Development of appropriate biosecurity safeguards to control movement in and out of facilities of pathogens and other adverse biological effects (e.g., Tiersch and Jenkins 2003).
4) Development of functioning storage repositories, with rules for use and disposal of samples, and with appropriate security for basic services (e.g., electricity, liquid nitrogen, refrigeration capabilities, and aeration for aquaria and holding tanks).
5) Implementation of archival-quality labeling of samples and the creation of robust databases capable of handling biological information concerning samples (including geographical information system (GIS) data on collections), and maintaining correct inventory and identification of sample locations.
6) Further development of capabilities computing and information transfer including the ability to interact and exchange information with other databases such as the Genetic Resources Information Network of the USDA (*www.ars-grin.gov*).

7) Increasing of sample processing capabilities to enable high throughput of samples. This would include installation and use of automated or semi-automated equipment for labeling, filling, and sealing of straws, and the procurement of commercial-scale freezing and storage capabilities.

8) After central facilities have developed strong operational capabilities, a sustained effort should be made to develop cooperation with other organizations and facilities. These relationships can include sharing of samples, capabilities and expertise. Efforts should be made to link cryopreservation with existing or planned activities such as fish sampling programs or cooperation with specialized hatcheries during spawning seasons.

9) To assist interactions among organizations, basic arrangements should be discussed and put forth as formal transfer agreements that can be negotiated and put in place to describe things such as responsibilities, rights, and ownership of samples.

10) Because different facilities will have different approaches there should be establishment of quality control protocols and standardization or harmonization of protocols, labeling, terminology, reporting of results, and databases. Development of Best Practices Manuals or other guidelines would assist these efforts.

11) Essentially, individual repositories can at this point be linked by establishment of a full repository system, and end users of cryopreserved sperm, such as hatcheries or farms could interact with this system.

12) Coordinated regional activities could take place in individual countries or be administered across borders by treaties or other agreements to encompass river systems or ecosystems.

13) Establishment of pricing structures, marketing, and business practices will be necessary for initial and continued commercial investment.

Conclusions

High-throughput technology has wide potential application. In biomedical science, it can preserve valuable strains for research purposes; in fisheries management, it can assist hatcheries in recovery efforts, and in aquaculture, it can enable and accelerate selective breeding and genetic improvement. High-throughput processing will serve as a fundamental technology for aquatic germplasm banking. These efforts will proceed more easily with large-bodied fishes or species that can provide copious volumes of sperm such as salmon, but can also proceed with small biomedical fishes such as zebrafish (Yang et al. 2007) or *Xiphophorus* (Yang et al. 2007, 2009) by pooling of samples to represent lines and increase the volume available for automated processing. Eventual development of processing equipment and containers that can rapidly handle small volumes (e.g., 10-50 uL) in a standard format or by use of microfluidic technologies would allow high-throughput of even individual small fishes or samples.

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