Thesis
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Hybridization and genetic manipulation in *Clarias* catfish

**A thesis presented for the degree of
Doctor of Philosophy to the University of Stirling**

by

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// ABSTRACT

The big-oui hybrid catfish (female *C. macrocephalus* x male *C. gariepinus*) was successfully achieved using artificial hybridization. This hybrid combines the superior taste of the *C. macrocephalus* with the faster growth rate and higher resistance to environmental conditions of the *C. gariepinus* and is now the biggest fresh-water aquaculture product in Thailand. In this thesis the results of experiments involving hybridization and genetic manipulation were used to investigate the nature of the hybridization event. This information was used to develop broodstock for *Clarias* catfish breeding programme.

Allozyme studies resolved 18 protein loci encoding different enzyme systems in *C. batrachus*, *C. macrocephalus*, *C. gariepinus* and the big-oui hybrid. *GPI-2**, *MDH-2** and *LDH-1** show clearly variation between the hybrid and the parental species and were used for species diagnostic loci. Comparisons of fertilisation and survival of the big-oui hybrid, reciprocal cross hybrid, F_2 hybrid, back cross hybrid and their parental species were carried out. The only cross involving the F_1 big-oui hybrid still gave viable embryos and fry was between female F_1 hybrid and male *C. gariepinus*. The F_2 hybrid never developed to hatch. Karyotyping studies showed a modal chromosome number of 2n=54 in *C. macrocephalus*; 2n=56 in *C. gariepinus*; 2n=55 in the big-oui hybrid, the reciprocal hybrid and the back cross hybrid. Using male *C. gariepinus* from two other stocks ('Malawee' and 'Wageningen') were carried out. to produce the big-oui hybrid. Cold shock at 2°C administered for 15 mins duration and 4 mins after fertilisation was the most effective in inducing 100% triploidy in big-oui

hybrid while heat and pressure shock were less effective. Growth performance of diploid and triploid hybrids was not significantly different. The triploid hybrid were shown to be functionally and endocrinologically sterile. Gynogenetic diploids were produced by fertilizing *C. macrocephalus* eggs with *C. gariepinus* sperm that had been genetically inactivated with ultraviolet (UV) light, and then cold shocking the eggs after fertilisation. The UV dose of 200 μWcm^{-2} for 2 mins using a sperm concentration of 2.5×10^8 ml^{-1} was optimal in genetically inactivating sperm without seriously compromising motility. Cold shock at 2°C, started at 4 mins after fertilisation and 15 mins duration gave the maximum number of meiotic gynogenetic offspring. The parental contribution in the gynogenetic offspring was check by using the species diagnostic allozyme loci and showed no evidence of male contribution. Gynogenetic offspring were grown on to investigate the sex ratio. All gynogenetic offspring were female suggesting female homogamety in this species.

The possible implications of the above results of hybridization and genetic manipulation studies in *Clarias* catfish culture are discussed.

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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

1.1.1 Aquaculture and World fisheries

The cultivation of aquatic organisms under controlled conditions is not new, but has benefited from man's technical progress during the last century which has turned the art of animal husbandry into a science. Fish farming has developed to the stage where the annual world production from aquaculture is over 22 million tonnes, which was over 20 per cent of the total aquatic harvest by fishing and farming in 1993 (FAO, 1994).

After the second world war, the global fish catch increased rapidly from less than 20 million tonnes to about 65 million tonnes, before coming to a virtual halt in the early 1970s. Figures published by the Food and Agriculture Organization (FAO) show that total aquatic harvest was 85 million tonnes in 1989. A combination of escalating fuel costs and declining wild fish stocks has resulted in a current annual growth rate of less than 8 % for the capture fish production worldwide, opposed with a current growth in world population approaching 2 % per year and the growing interest in the health advantages as a protein source compared with poultry and red meat. According to these various factors, it is no surprise that aquaculture is now making such a large contribution to world fish supplies (FAO: RAPA, 1989).

1.1.2 Aquaculture in Thailand

Fish farming or aquaculture in Thailand stems from the ancient practice of trapping

fish. During the rainy season (May-August) the southwest monsoon wind dominates and brings about heavy rainfall so that flooding occurs in different parts of the country. Because of its topography, central Thailand is the most flooded area. When flooding occurs, fish from the upstream-waters follow the flood along the canals into the lower land rice fields. Fish that enter into wetlands or small swamps in rice fields grow well and are caught for food consumption after the rice is harvested in the dry season.

Equally interesting are the fish that follow the flood along the streams and enter small ponds constructed and maintained by the farmers living along the banks of the wetlands. Those ponds, constructed for water holding purposes, are deeper than the adjacent canals and are connected with them by short narrow passages through which fish enter into the ponds at the time when the water level is high. Attracted by large quantities of fish caught from these ponds after the water is drained out, the farmers have constructed numerous small ponds for the purpose of trapping fish during the flooding season, or even in some places where canals and streams are filled with water throughout the year. At intervals of about 2-4 months, the ponds are blocked and water is drained out and the fish are caught with simple hand nets. Operations of this kind may be regarded as an origin of fish culture.

The foregoing simple trapping method was steadily improved and gradually developed from trapping-holding to trapping-holding-growing, and finally into complete husbandry practices when people learned how to collect fingerlings or produce fry for stocking.

At present, aquaculture is widespread in Thailand with well developed technology, as indicated by increasing of the average annual production (table 1.1) and increasing of farms and culture areas (table 1.2). The annual aquacultural production reported in 1992 was 371,400 metric tonnes, valued at 29,712.7 million Baht (25 Baht=1US$).

This tonnage is about 12 % of the combined total catch and aquacultural yield of the entire country. Accounting for most of the aquacultural production are some 78,473 aquaculturists whose pond surface areas range from less than 1 rai (1 rai=1,600 m^2) to some 300 rai. The total water surface involved is about 308,134 rai (47,405 ha), a figure that is estimated at only 2 % of the total potential area of the country (Department of Fisheries, 1994).

Table 1.1 Fisheries production of Thailand in quantity and by sub-sectors, 1988-1992 (Department of Fisheries, 1994).

Type	CAPTURE		CULTURE		TOTAL	
Year	Quantity 1,000 tonnes	Value M.Baht*	Quantity 1,000 tonnes	Value M.Baht	Quantity 1,000 tonnes	Value M.Baht
1988	2,418.7	21,607.7	211	10,814.8	2,629.7	32,422.5
1989	2,479.6	22,163.4	260.4	13,706.8	2,740.0	35,870.0
1990	2,489.4	24,040.1	297	17,355.6	2,786.4	41,395.7
1991	2,614.6	29,694.5	353.1	23,331.3	2,967.7	53,025.8
1992	2,868.4	35,831.8	371.4	29,712.7	3,239.8	65,544.5

* Approx. 25 Baht= 1 US$ or 40 Baht = £1

Of all the operations of fish farming in Thailand, pond fish culture recieves the most attention. This kind of operation can be conducted under controlled conditions and yields high production if properly managed. It is, therefore, extensively practised in different parts of the country, mostly around the Bangkok area and the central area of the country in which good transportation and markets are available. Predictable values as food habits, feeding frequencies, stocking rate, stocking size and species.

Table 1.2 Number of farms and area (rai) of fresh-water farms in Thailand (Source: Department of Fisheries, 1988-1992).

Type	Pond Culture		Paddy Field Culture		Ditch Culture		Cage Culture		Total	
Year	Farms	Area	Farms	Area	Farms	Area	Farms	Area	Farms	Area
1988	66965	175340	8257	149164	847	1938	904	50	76973	326500
1989	68972	159837	8420	149372	806	1660	935	82	79133	310952
1990	75310	160354	8536	148495	826	1783	891	134	85563	310766
1991	86491	160783	8976	152375	1002	1926	865	127	97334	315211
1992	108356	201672	9440	159488	1286	2332	760	114	119842	363606

1 rai = 1,600 m^2

1.1.3 Catfish production in Thailand

Fresh-water catfish especially *Clarias spp.* are widely distributed in southeast and south Asia and Africa. They exist in a variety of habitats from brackish water in estuaries and mangroves to fully freshwater rivers and lakes. Recently, they have become a popular food fish and subsequently become popular species for culture because they can be spawned, grow very fast, can tolerate farming conditions and are easy to culture.

In Thailand, *Clarias* catfish are now one of the most important cultured fish (production of 29,136 tonnes in 1991) (FAO, 1994). The names of the fish that have been most commonly propagated in freshwater ponds, along with their draft synopsis of aquaculture practices, are listed in table 1.3. The catfish in Thailand mostly belong to the family *Clariidae*, of which five species are present, *Clarias meladerma* Bleeker, *Clarias batrachus* (Linnaeus), *Clarias teymanni* Bleeker, *Clarias leiacanthus* Bleeker and *Clarias macrocephalus* Gunther, (Smith, 1945). The common name *"walking catfish"* in English, or *"pla duk"* in Thai, is a generic name for a numer of species belong to the family *Clariidae*. The two most economically important native species in Thailand are *Clarias batrachus* and *Clarias macrocephalus*, locally known as *"pla duk dan"* and *pla duk oui"*, respectively. The fry of *C. batrachus* are easily obtained from the spawning pond. Unfortunately, *C. macrocephalus* does not readily reproduce in captivity. However, they can be induced to breed if injected with gonadotropic hormones. An interesting fact about culturing catfish in ponds is the feeding of *C. batrachus*, with a mixture of fresh ground trash fish (90%) plus rice bran (10%). This kind of feeding is done by commercial fish farmers whose farms are located near the central area where trash fish can easily be obtained. The average production is 60 to 90 metric tonnes per ha when the fish were stocked at the rates of 100-180 fry per m^2 of pond surface area. In recent time, culturing also revealed that catfish can successfully and commercially be fed with dry

floating pellets. Thai consumers have a preference for *C. macrocephalus* but due to limitations in fry availability and slow growth, its culture is still limited in comparison to that of *C. batrachus*.

In 1987, another species of catfish, *Clarias gariepinus* (Burchell, 1822), was introduced to Thailand from Laos. This species was distributed and cultured in the northeast region and some provinces in the central region. The local people called it "*pla duk Russia*" because it had been introduced to Laos by the Russians. Studies on this species have been carried out at the National Inland Fisheries Institute, Department of Fisheries since May 1988. *C. gariepinus* is the most important of the 32 African catfish species for aquaculture because of its size, fast growth, omnivoracity, resistance to extreme environmental conditions and because it can be spawned using relatively simple artificial reproduction techniques. The preliminary studies in Thailand showed that *C. gariepinus* grows very fast in earthen ponds on Thai fish farms and can be spawned by hormone induction.

Furthermore, the breeding of the hybrid catfish was achieved by a team of fishery biologists from the National Inland Fisheries Institute by using female *C. macrocephalus* and male *C. gariepinus*. The hybrid shows faster growth than *C. macrocephalus*, has high resistance to environmental conditions (similar to *C. gariepinus*) and intermediate morphological characteristics and meat quality compared to the parental species. The hybrid between female *C. macrocephalus* and male *C. gariepinus*, named "*pla duk big-oui*" has become increasingly important and generated high demand in the markets. From 1988 until 1991, the annual production grew from nothing to an estimated over 20,000 metric tonnes. In the culture ponds, the hybrids can grow up to marketable size within 3 months which means the farmers can produce at least 3 crops per year. This is an advantage in animal protein production.

Table 1.3 The most commonly cultivated fresh-water species in Thailand and their production in the whole country by type of culture in 1991, Quantity : metric tonnes, Value : 1,000 Baht (Source : Department of Fisheries, 1992).

Culture	Pond culture		Paddy field		Ditch culture		Cage culture		Total	
species	Quantity	Value	Quantity	Value	Quantity	Value	Quantity	Value	Quantity	Value
Clarias spp.	28,883	721,258	249	13,843	3	63	1	12	29,135	735,176
Ophiocephalus spp.	3,154	129,352	1,222	48,980	1,190	45,264	1,630	49,648	7,196	273,245
Pangasius suchi	13,524	152,363	-	-	-	68	987	15,847	14,518	168,277
Puntius gonionotus	14,363	281,026	1,814	38,092	93	1,475	5	127	16,274	320,720
Oreochromis niloticus	22,406	336,356	5,453	56,465	244	2,700	3	72	28,106	395,593
Cyprinus carpio	2,106	49,143	366	8,938	3	67	-	-	2,475	58,148
Chinese carp	838	12,777	319	3,205	-	-	-	-	1,157	15,983

The increased awareness that fish is a healthy source of protein has further driven demand of more fish worldwide. The Thai Department of Fisheries plans to develop the culture system of catfish and tilapia to produce fish for export (especially as a fillet product). Although the hybrid catfish has a fast growth rate in the early stage (growing up to an average of 300 g in 3 months), after this the growth rate decreases. Production of individual fish of one kilogramme (for making fillets) needs a grow out period of one year. Larval survival of the hybrid is lower than in intraspecific *C. macrocephalus* batches, whilst survival in the reciprocal hybrid cross (female *C. gariepinus* X male *C. macrocephalus*) is almost zero. These two main problems create the need to investigate ways of increasing the growth rate and early stage survival rate in these hybrid crosses.

1.1.4 Genetic aspects for aquaculture and fisheries management

The causes of the variations that exist for the phenotypes and the way that the differences are inherited is the science called genetics. Breeding is the applied science of genetics that exploits the heritable component of variation in order to change the population for man's benefit.

The study of genetics for aquaculture is relevant to the aquatic environment and all of man made impacts on the aquatic environment. Direct implications are obvious for fish cultivation in all its many forms, but the genetic consequences of exploitation by commercial fishing must also be considered, and any habitat change must also have its genetic consequences. Form and function are amalgams of the inherited potentialities of organisms and the environmental constraints applied to them, and change in either has a genetic consequence.

Basic breeding concepts have only recently been applied to aquaculture. Fish culture lags far behind other areas of animal husbandry in that fish culturists raise animals

that either come from wild stocks or are only a few generations removed from the wild, few domesticated stocks of fish exist. Because aquaculture genetics is comparatively new, but especially because most aquaculturists feel that genetics and geneticists are incomprehensible, genetic aspects of fish husbandry have had comparatively little impact on productivity and profits, except in the ornamental fish industry. However, several breeding programmes have been developed and are being used. Despite the fact that breeding has had minimal impact on improving productivity in the aquaculture industry, it is imperative that aquaculturists have a good grasp of genetics and breeding principles because they are among the major factors that govern productivity. One reason why it is imperative that farmers understand the basic principles governing genetics and breeding is the unfortunate fact that some effort is often needed simply to maintain the situation as it was before. It is also unfortunate that inattention to the genetic aspects of fish farming can actually cause productivity to decline as inbreeding or other genetic consequences of mismanagement result in slower growth, decreased viability, decreased disease resistance, and decreased egg production.

To date, most effort in fish culture has been directed toward improved diets, health management, and water quality management. As important as they are, these disciplines deal with the environment in which fish live, and improvements in these areas simply improve the environments. The aims of aquaculture are more economical as to raise the fish that have a faster growth rate, greater dressing percentage, lower food conversion, and greater disease resistance. Because of this, one of the goals of hatchery management should be to incorporate basic genetic and breeding concepts into routine hatchery management in order to maximize the productivity potential of the fish.

1.1.5 Genetic researches need for improvement of commercial fish stocks

Perpetuation of the resource is the common goal of all fisheries management programmes. Genetic factors affect this goal because fish are the product of their genes, the environment, and of the interaction between the genes and the environment. The genetics of fish, in connection with the environment, determine the quality and persistence of the fishery resource. Fisheries managers must realize that implementation of regulations, stocking strategies, and other management activities affect the genetic make-up of fish stocks. Management activities that impact the genetics of fish stocks include: 1) maintenance of a fishery with adequate natural reproduction, 2) enhancement of a fishery with marginal or inadequate natural reproduction by stocking, 3) rehabilitation of a depleted fishery by stocking or control of harvest, and 4) maintenance of a input and uptake fishery.

Hatchery management should, in general, try to preserve genetic variation in breeding populations while producing fish that are suitable for their intended use. The principal concerns are: 1) the hatchery practices may detrimentally alter survival, yield, or reproduction, and 2) small effective population sizes in hatcheries can lead to inbreeding and loss of genetic diversity.

Concerns about the genetic impacts of hatchery management depend on the use intended for the hatchery fish. Hatchery bred fish may be stocked in an aquacultural facility for food production, in a natural environment, or in some captive setting as broodstock. The reproductive fitness of fish destined for slaughter is not important; in fact, reproduction is often undesirable. In contrast, the fitness of fish used as broodstock or for stocking in natural environments is of paramount importance because the fitness of future generations depends on genetic characteristics of the present generation. Improvements to the management of genetic resources in hatcheries will increase the benefits due to stocking hatchery fish.

1.1.6 "Genetic Manipulation" the new technology in aquaculture

At present, most of fish propagation is under artificial conditions. The techniques of artificial insemination developed, make it possible to manipulate the sperm, egg, and zygote in different ways so that the genetic constitutions of the offspring populations are changed dramatically. The genetic manipulation techniques recently developed for fish species can be categoried as shown in Table 1.4.

Chourrout (1987) described how the production of individuals whose genomes originate from only one parent is permitted by three types of manipulation:

1. Self fertilisation, which requires the development of induced hermaphrodites in fishes, because most cultivated species are gonochoristic.

2. Induced gynogenesis, which also involves two parents; the sperm is inactivated by radioactive rays or UV light, its genomes is destroyed prior to fertilisation. The genome of the embryo is provided by an intact egg, and it can be doubled by suppression of the first meiosis or the first mitosis.

3. Induced androgenesis, which also involves two parents; the egg is only a host for development, because its genome is destroyed prior to fertilisation. The genome of the embryo is provided by an intact spermatozoa, and it can be doubled only by suppression of the first mitosis.

If the genomes of both parents contribute to the development, treatments blocking meiosis or mitosis give rise to two kinds of polyploids:

1. Triploids resulting from the fusion of diploid female and haploid male nuclei. The suppression of meiosis II induced after fertilisation of gametes from two different species similarity provides triploid hybrids.

2. Tetraploids resulting from the doubling of the first diploid nucleus in the embryo suppression of the first mitosis.

Table 1.4. The results of different manipulation techniques on the sperm used for fertilisation and on fish zygotes (modified from Nagy, 1987)

Sperm	**Eggs**	**Zygotes**	**Zygotes**
Treatments	*intact*	*early shock*	*late shock*
intact	normal diploid	heterozygous triploid	heterozygous tetraploid
genetically inactivated	haploid gynogenetic	heterozygous diploid gynogenetic	homozygous diploid gynogenetic

Sperm	**Eggs**	**Zygotes**	**Zygotes**
intact	*Treatments*	*early shock*	*late shock*
intact	*genetically inactivated*	-	homozygous androgenetic

Finally, the addition of foreign genes carried either by active chromosome fragments to the genome of the species, or by cloned plasmids through gene transfer are the new techniques applied to confer new chacteristics to the aquatic animals.

The genetic inactivation of sperm is usually achieved by administering large doses of ionizing or UV radiation. After radiation has completely destroyed the chromosomes, the spermatozoa retain the ability to penetrate into the eggs; the cell division they initiate proceeds normally.

Experiments to increase ploidy have been developed for many fish species. Usually these are sublethal temperature treatments, either cold or heat shock of the zygotes, but high pressure treatment of zygotes is also very effective in some species. Depending on the timing of these physical shocks, the mechanisms of diploidization are different. The shock applied shortly after fertilisation causes the retention of the second polar body. Thus, in the case of gynogenesis, it results in the union of two haploid complements produced in the second meiotic division. Late treatments are

applied shortly before the expected time of first cleavage, and they therefore prevent spindle formation, resulting in endomitosis. The ploidy of the embryos is doubled after these shocks. It is easy to understand that completely homozygous, diploid, gynogenetic offspring can be produced by late shocks applied to haploid, gynogenetic zygotes. Similarly, late shocks applied to normal diploid zygotes result in tetraploid fish.

Genetic manipulation, if incorporated with hormone sex reversals, allows us to produce monosex, usually all female populations, which have advantages over the bisexual populations of numerous species. Other possibilities arise from the combination of sex reversal with gynogenesis or tetraploid production to alter the genetic constitutions, the sex and ploidy, of their offspring populations. In future, we can expect the appearance of new techniques to manipulate the genome of the species and we will able to design the genomes of farm fish according to our needs to produce required disease resistance and special characteristics.

1.2 Literature review

1.2.1 Biology of *Clarias* catfish use in this thesis

African species: *Clarias gariepinus* (Burchell, 1822)

Early in the twentieth century, colonists realised that *Clarias* might have an economic value since they were highly prized by local inhabitants and wild catches demanded high market prices. By the early 1950's Belgian workers started cultures of *Clarias* in the former Belgian Congo. The results were disappointing compared with those from *Tilapia* culture. Even though *Clarias* was considered as an alternative fast growing productive protein source, due to the unsatisfactory state of taxonomy, in part, it was often not known which species is being reared (Teugels, 1986). Lack of basic

information on the biology and taxonomy of the species in this region made it difficult to overcome these problems.

The catfish is now widely accepted as a most distinquished candidate for aquaculture. The attributes of *C. gariepinus* of relevance to its culture include: wide native distribution, ability to utilize atmospheric oxygen, high consumer preference ranking, suitable reproductive strategy, favourable nutritional efficiency and feeding habit, fast growth rate, tolerance of environmental extremes, resistance to disease and tolerance of high density culture (Hayler, 1993).

Morphological identification in *C. gariepinus*

The head is somewhat between rectangular and pointed in dorsal outline; the snout is broadly rounded. The eyes have a supero-lateral position and are relatively small. The four pairs of circum-oral barbels show a negative allometric growth: small specimens have relatively long barbels, while the barbel length decreases in larger fish. The oval occipital fontanel partially invades the occipital process. The distance between the occipital process and the base of the dorsal fin is short. The dorsal fin almost reaches the caudal fin. An adipose fin is absent. The original of the anal fin is closer to the base of the caudal fin than to the snout; it nearly reaches the caudal fin. The pelvic fin is closer to the snout than to the caudal fin base. The pectoral spine is robust, serrated only on its outer face, the number of serrations increasing with age. The pectoral fin extends from the operculum to below the first dorsal fin rays. The lateral line appears as a small, white line from the posterior end of the head to the middle of the caudal fin base. The openings to the secondary sensory canals are clearly marked. There are two patterns of body coloration; the uniform and the marbled pattern. In the former, the dorsal surface and the flanks of the body and the dorsal parts of the pectoral and the pelvic fins are generally dark greyish-greenish black, while the belly and the ventral parts of the paired fins are lightly colored. In the marbled pattern, the fish show

irregular dark blotches on a light colored background above and laterally. The belly and the ventral parts of the paired fins are whitish. A series of light and dark bands may occur on the caudal fin, the proximal third of the fin is lightly colored while its other part is dark (Boulenger, 1911; Bell-Cross, 1976; Teugels, 1986).

Thai *Clarias* species: *Clarias macrocephalus* Günther and *C. batrachus* (Linnaeus)

The *Clariid* catfish are of great interest because they have, in addition to gills, an accessory breathing organ occupying the upper part of each branchial cavity. These organs, having an arborescent shape, enable the fish to breathe atmospheric air. The catfish of the genus *Clarias* are among the most abundant, most widely distributed, and most economically important of the fresh-water fishes of Thailand. They are eaten extensively in the households of fishermen and farmers, and are regularly offered for sale in the markets of Bangkok and other communities throughout the country. Fish are exposed for sale alive in baskets, in shallow tubs with little or no water, or on stone slabs, like *Anabas* and *Ophiocephalus*, and if not sold in a day they are taken back to market the next day. The fish have a great reputation for their wholesome qualities, and are in special demand for convalescents and invalids (Smith, 1945).

The common name *"walking catfish "* in English, or *"pla duk"* in Thai, is a generic name for a number of species belong to the familly *Clariidae* which five species have been ascribed to Thailand but only two species can be definitely accredited on the information now available. The two most economically important species are *Clarias macrocephalus*, locally known as *"pla duk oui"* and *Clarias batrachus*, known as *"pla duk dan"*.

Spawning in the wild occurs during the rainy season, from May to October. *C. batrachus* spawns in small horizontal holes of 25 cm in diameter and about 30 cm deep in the earthen bank 30 cm under water surface. About 2,000-5,000 yellowish-brown eggs are laid at a time. The *C. macrocephalus* female makes a small round nest with grassy bottom. The eggs are deposited in the nest and attached to the roots of aquatic vegetation. The male will take charge of these eggs until they are hatched out. A female weighting 300-500 g can produce between 5,000-10,000 eggs.

They are nocturnal and can live at very high stocking density because of their air breathing capability. They are usually found in canals, swamps and paddy fields. In the wild, the fry feed on protozoa and small crustaceans, and when they grow bigger they feed on worms, insects and organic matter at the bottom of the pond. As a food, both species are the commonest and commercially the most important of the Thailand clariids. *C. macrocephalus* is rated higher than *C. batrachus* because its flesh is richer, better flavored, and more nourishing, according to the popular opinion.

Morphological identification in *C. macrocephalus* and *C. batrachus*

The native species, *C. macrocephalus* is closely related to *C. batrachus*. The species can be morphologically distinguished by the shape of the occipital process, which is more pointed in *C. batrachus* and evenly curved in *C. macrocephalus* (Figure 1.1). The occipital process extends close to the dorsal fin.

The body is elongate with head broadly depressed, four pairs of well developed barbels and small eyes. The nasal barbels reach to or beyond the gill openings, maxillary barbels extend to the middle or tip of the pectorals, vomero-palatine teeth are obtusely conical and form a crescent-shaped band, which in its center is broader than the intermaxillary band, and there is a nearly smooth pectoral spine.

Genetical identification

The taxonomy of catfishes of the genus *Clarias* (*Clariidae*) has for a long time been very confusing and only recently have detailed systematic revisions become available, enabling the correct identification of the species (Teugels, 1986). The foregoing monographs are based on analyses of morphological and osteological characters of large collections of fishes, and may be regarded as a classical taxonomic approach. In some cases, however, problems in identification remain unresolved. Teugels (1984) used a new systematic revision of the 120 nomeinal African *Clarias* species to recognises only 32 valid species. He also clarified the systematic status and the more important synonyms of *Clarias gariepinus* and *C. anguillaris,* two species of considerable importance in aquaculture. Other, more recent techniques, such as cytology and electrophoresis, have lately been applied to better effect than the classical approach. Several papers have reported efficient electrophoretic methods for species identification; these methods prove useful in strain identification and also in hybrid recognition (Teugels, *et al.*, 1992b). The Clariidae and the Ictaluridae represent the best studied families. The systematic status of a number of species and families has been either elucidated or confirmed by genetic approaches. Duplication of ancestral genes occurred in catfishes just as in other vertebrates. The genetic structure of and gene flow among natural populations have been documented in relatively few cases, while the evaluation of strains for aquaculture is in progress (Volckaert and Agnese, 1995).

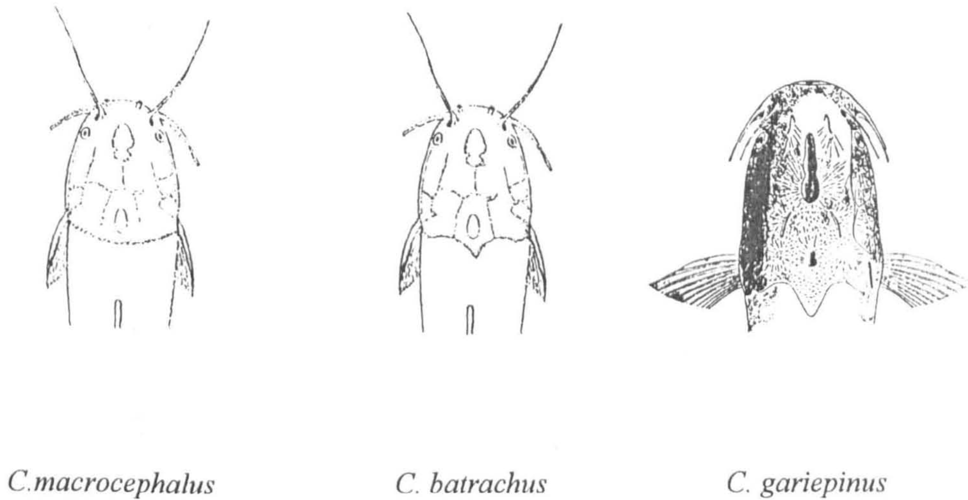


Figure 1.1 The occipital process of *C. macrocephalus* (left), *C. batrachus* (centre) and *C. gariepinus* (right) (Boulenger, 1911; Smith, 1945)

1.2.2 Recent studies in fish genetics.

Fishery biology has much to gain from the advances in technology that now permit the study of gene products and of components of genes themselves. Several of the most important questions in fisheries, e.g., those of stock assessment and biochemical identification, can be addressed readily through the study of genetic variability within among populations of fishes (Whitmore, 1990). The geneticist can explained how the genetic material is transmitted from generation to generation via the gametes (sperm and eggs). The basic genetics have information that shows how the genetic information passes from parents to offspring. The facts about genes and chromosomes; DNA and protein synthesis; cell divisions (mitosis and meiosis), and sex determination are clearify investigated. This information builds up the basic laws of genetics, particularity those dealing with molecular genetics and cytogenetics, since the laws of inheritance were established by Gregor Mendel, an Austrian monk who

studied how seven morphological differences in the garden pea are inherited (Purdom, 1993; Tave, 1993).

Genetics has been defined as the study of differences among individuals. If all the individuals in a particular species were identical, we could still study their morphology, physiology, ecology, etc. The study of inheritance depends upon finding individual differences so that the similarity of parents and their offspring can be compared relative to the similarity among unrelated individuals (Allendorf and Ferguson, 1990).

Genetic variability can be thought of as existing at two levels: (1) genetic differences between individuals within populations, and (2) genetic differences between populations within the same species. The first level is investigated by the traditional Mendelian genetics. However, the study of how individual variability becomes transformed into differences between populations is fundamental to the study of evolution (Naevdal and Dalpadado, 1987)

Fish genetics is of relevance to fish breeding, whether in the context of fish cultivation or the exploitation of fish by fishing (Krasznai, 1987; Purdom,1993).The genetics of fish interacts with their culture in four main areas of study: (1) the quantitative genetics, hybridization and inbreeding, which are approached at the organismal and phenotypic level; (2) ploidy manipulation which the advances made by using the techniques of exploring the chromosomal level; (3) single-locus allozyme studies which have increased our understanding of the genetic consequences of bring wild populations under domestic control and of releases of cultured stocks into the wild; and (4) the application of recent DNA techniques to the development of gene transfer in fish (Kapuscinski and Jacobson, 1987; Tave, 1993; Beaumont, 1994).

Quantitative genetics: traits of importance of aquaculture are generally controlled by a number of genes and are therefore best studied by quantitative genetic techniques. From the commercial point of view, artificial selection needs to be carried out to enhance those features or traits which increase commercial potential or to instigate the process of domestication (Kapuscinski and Jacobson, 1987).

Hybridization and inbreeding: the existence of hybrid vigour, or heterosis and inbreeding depression have been recognized for a long time. The degree of differentiation between strains required to produce measurable hybrid vigour is an important criterion. Very closely related strains may produce none while more distantly related groups may suffer from outbreeding depression, possibly brought about by the break-up of coadapted gene complexes or other changes in genetic architecture (Purdom, 1993). Hybrids between salmonid species tend not to show measurable hybrid vigour and have little advantage in fish farming when compared with *S. salar* (Chevassus, 1979). Nevertheless, it is still important to explore interspecies hybridization in any new fish species with aquaculture potential (Chevassus, 1983). In practical ways it may be advantageous to breed for ease of capture and this is the case with catfish which are grown for angling. Dunham *et al.* (1990) reported that the hybrid cross between channel catfish, *Ictalurus punctatus*, and the blue catfish *I. furcatus*, was more vulnerable to angling than either parental species and showed heterosis for growth rate when stocked at high density.

Interspecies hybridization can be used in tilapine fish to produce all male offspring, a valuable feature because a major constraint on tilapia production is their uncontrolled reproduction in grow-out ponds (Hulata *et al.*, 1983). Interstrain crosses in *Oreochromis nilotica* can produce significant heterosis in both F_1 and F_2 generations (Tave *et al.*,1990)

In contrast to the rather varied success in the search for hybrid vigour in interstrain or interspecies crosses in aquaculture species, the deleterious effects of inbreeding are usually strongly expressed even in the first inbred generation (Gjedrem, 1992; Tave, 1993).

Ploidy manipulation: the ability to manipulate the ploidy of aquaculture organisms was initially brought to prominence by Purdom (1969) who was working on fish, and the technology was only later takén up for use on molluscan shellfish and amphibians. During the 1980's many fish and shellfish species have been the subject of ploidy manipulation and these developments have been reviewed by Thorgaard and Allen (1987); Thorgaard (1992) and Tave (1993). Triploid and gynogenetic diploid fish are valuable for aquaculture. The most important feature of triploids is their sterility. Homologous chromosomes in the germ cell of triploids cannot synapse during early meiosis I and normal gametogenesis does not occur. Thus energy usually diverted to gamete production in mature fish is available for somatic growth in triploid. In fish the undesirable side effects of sexual maturation, such as high mortality, reduced meat quality and slower growth, are not always eliminated in male triploids though they are functionally sterile. Gynogenetic diploids have two important features of relevance to aquaculture. First, in fish which exhibit female homogamety, such as carp and many salmonids, all gynogenetic offspring should be female (XX). The second important aquaculture potential for gynogenesis is its allows the possibility of rapid development of inbred lines for domesticated hatchery broodstocks (Beaumont,1994).

Allozyme genetics: the development of the techniques of protein allozyme electrophoresis over the last 25 years has enabled allozyme data to be used to address a number of questions of importance in aquaculture. There are three important aspects of fish or shellfish hatchery practice which are likely to have a significant impact on genetics of culture species, and all can be investigated using allozyme data. First, if the number of progenitors is small then genetic drift will lead to loss of rare alleles

and a reduction in heterozygosity. Second, inbreeding, and consequent inbreeding depression, will occur if the offspring of the original broodstock are retained as new broodstock. Third, cultured aquatic organisms are protected from some aspects of natural selection but are subjected to artificial selection for size and other aspects of culture as an unintentional result of normal husbandry procedures (Allendorf and Ryman,1987; Tave,1993).

Transgenic studies: The principal involvement of DNA technology is currently in transfection, the introduction of novel genes into aquaculture organisms. There are many potential benefits of this approach, including, for example the development of disease resistant stocks, faster growth animals or freeze resistant stocks. Fish are currently a valuable group of animals for application of gene technology and trangenic induction because ethical considerations which tend to constrain the use of genetic manipulation in mammals and other higher animals are less likely to apply to fish involved in aquaculture. Transgenic fish have been developed with a number of uses in mind, and as the technology develops, more uses can be envisaged. Woodwark, et al. (1994) reported that the literature lists 40 constructs used in attempted genetic modification of fish. Of these 40 constructs, 25 are reporter genes, mostly of bacterial and viral origin, one is the antifreeze gene from winter flounder and the other 14 are growth hormone constructs. Enhanced growth rates have been reported in several fish species used in aquaculture (e.g. common carp, Atlantic salmon and channel catfish). The increase in growth rate achieved by genetic modification has not been predictable. It is expected that experimentation with different types of gene construct will be necessary to determine the appropriate level of expression of growth hormone. The role of growth hormone in promoting faster growth is not fully understood. Du et al. (1992) reported that the fastest growing Atlatic salmon contained the lowest amounts of growth hormone in blood plasma, although in general, there was no significant difference between the plasma GH levels between transgenic fish and controls. The principal methodology for introduction of

novel DNA into fish is by microinjection of a solution containing around 10^6 - 10^8 copies of the DNA into the eggs following insemination but before first cleavage (Maclean *et al.*, 1987; Penman *et al.*,1987a).

1.2.3 Hybridisation in fish.

The interbreeding of different taxonomic groups has been attempted in many domesticated animals and plants. Among animals, fish are very well suited to this approach and a vast literature exists on artificial hybridization, even greater than that for natural hybridization. Moreover, the scale and range of studies in artificial hybridization is very much wider than that of natural events (Krasznai, 1987).

The importance of natural hybridization lies not so much in its positive implications for fish breeding, although artificial hybridization is a powerful tool in fish culture, but more in its implications for evolutionary relationships. Hybridization in the natural environment is more relevant today as an environmental issue in the sense that introduced, or exotic, species might interbreed with and upset the natural genetics of native populations of fish. This problem is widely known as part of the trouble arising from the introduction of non-native species (Chevassus, 1983).

There are four basic ways of achieving artificial hybridization. The most natural way is the simple cohabitation in captivity of males of one species and females of the other. This is the usual way for aquarium species to be crossed, but pond or farm fish such as the tilapias can also be brought to a successful spawning with this approach. In one sense, this method is very close to natural hybridization brought on following introduction of a new species into the habitat of another. A further development of natural cohabitation is to use hormone stimulated sexual maturation, usually done by injection of the fish with extracts of pituitary gland, often on an empirical basis. This

method is suitable for unreliable spawners such as some species of catfish and carp (Krasznai, 1987).

Artificial fertilization, whereby eggs and milt are hand stripped from the fish for mixture, is the most practical way of producing hybrids or, indeed, producing any genetic cross. It is widely practiced with many fish species and may need stimulation by gonadotropic hormone prior to hand stripping as for example with clariid catfish which are the subject of this thesis and where the two species differ in size and natural period for ovulation and spermiogenesis (Longwell, 1987).

The spread of the artificial propagation techniques has made more crossing and hybridization works possible. No accurate data concerning artificial and natural hybrids are available, but their number can be estimated at perhaps 5,000 to 6,000 (Chevassus, 1979) and there is an unlimited potential in this field. Krasznai (1987) stated that it is merely a technical problem to develop a new "product" i.e. a hybrid with genetic manipulation. Close genetic relationship naturally renders the possibility of successful crossing more feasible.

More logical approaches to hybridization address specific problems such as the clarification of taxonomic relationships, the development of experimental tools for studying physiology, the production of genetic variance for selection programmes, the control of sex ratio and the production of superior fish for aquaculture purposes.

Krasznai, (1987) classified some of the most important aspects from interspecific hybridization as following :

-combination of different genetic features

-development of new properties

-changing etology

-development new feeding habits

-producing new heterosis hybrids to increase productivity

-enlarging the structure of the fauna

-producing infertile stock

-development of monosex populations

-inducing polyploidy

-inducing gynogenesis or androgenesis.

The production, by hybridization, of new forms of fish which are more suited to specific needs is by far the most purposeful approach in this branch of fish breeding. In many cases, however, the aspiring breeders simply hope that a hybrid will perform better than the parents in general terms, i.e., in exhibiting hybrid vigour. This approach is seldom rewarded. Most of the fish hybrids which have been produced in the last ten centuries are significantly less fit than the parents and are of little practical value. In most cases, the hybrids show intermediate inheritance, poor viability and sterility, and have limited practical potential (Purdom, 1983; Chevassus, 1983; and Longwell, 1987).

Chevassus (1983) in his study of interspecific hybridization mentioned all the possible products that may arise from the hybridization of two species (Table 1.5). The progeny might be haploid or diploid gynogenetic, haploid or diploid androgenetic, or diploid, triploid or tetraploid hybrid. Which of will actually develop depends upon the genetic combination of the parental gametes of the two different species. He also described two main types of results from hybridization:

1) parthenogenetic development, gynogenesis or androgenesis, according to the origin of the genetic stock;

2) development of hybrid diploid, triploid, or tetraploid genomes, according to the ploidy of the parents.

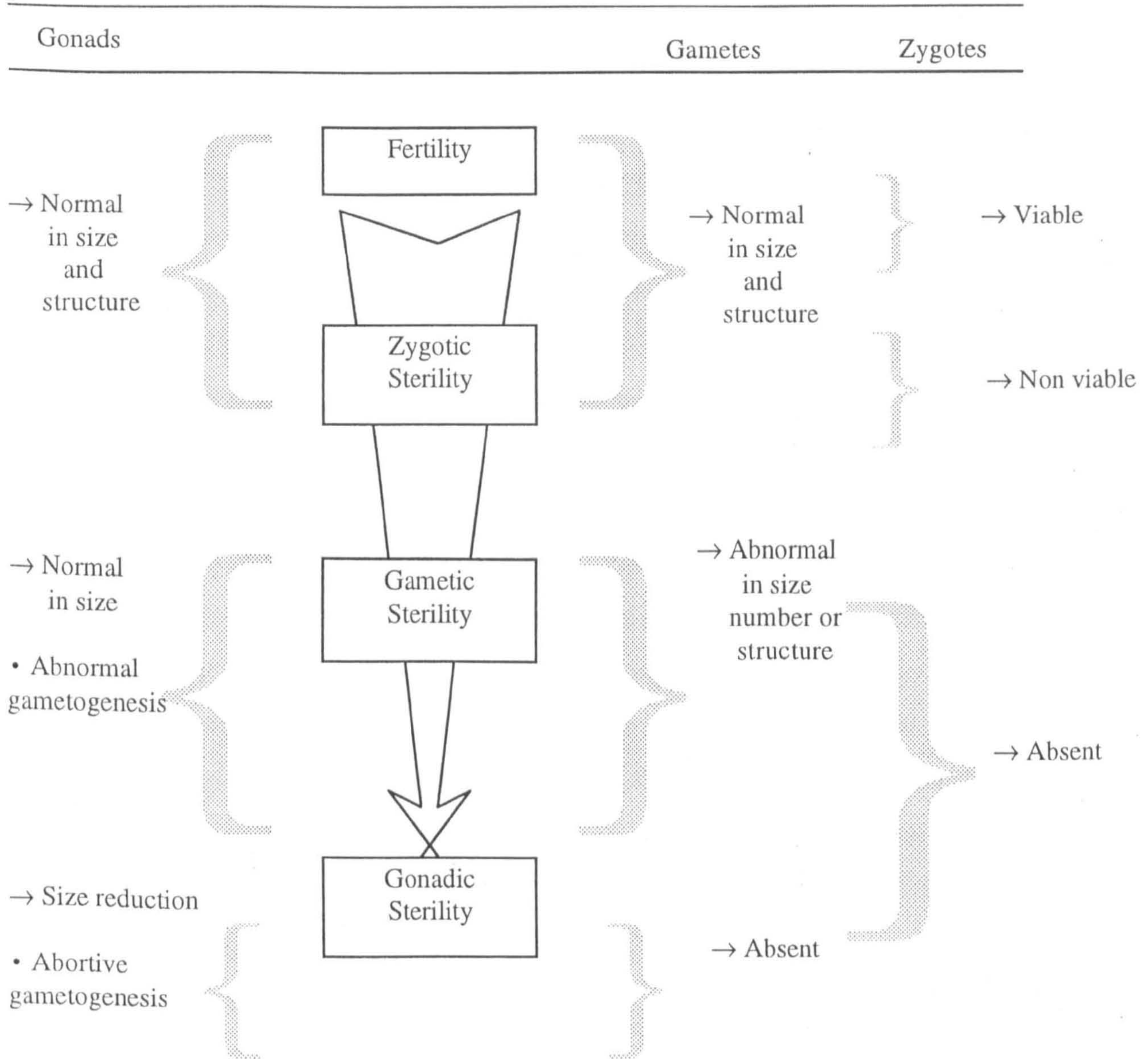
Table 1.5 Possible results of a hybridization, according to the nature and ploidy of the parental genetic input (modified from Chevassus, 1983).

Male Female	O (Elimination)	Na (Haploid pronucleus)	2Na (Diploid pronucleus)
O (Eliminate)	--	Haploid androgenetic Na	Diploid androgenetic 2Na
Nb (Haploid pronucleus)	Haploid gynogenetic Nb	Diploid hybrid Na + Nb	Triploid hybrid 2Na +Nb
2Nb (Diploid pronucleus)	Diploid gynogenetic 2Nb	Triploid hybrid Na + 2Nb	Tetraploid hybrid 2Na + 2Nb

The types of sterility in hybrids were also summarized by Chevassus (1983) as showed in Table 1.6. There are reports stating that some artificial hybrids are fertile, e.g., tilapia hybrids (Pruginin *et al.*,1975; Hussain, 1992), carp (Hume *et al.*,1983), channel catfish (Scott and Crossman, 1973) and catfish *Clarias gariepinus* X *Heterobranchus longifilis* (Teugels *et al.*, 1992a).

The conclusions of hybridization remain largely valid but with some qualification. In the majority of cases, hybrids are intermediate for those characteristics which normally distinguish the parents, but a bias towards one parent or the other is very common. This non-additivity of inheritance is compatible with the concepts of developmental homeostasis and with the seemingly low levels of additive genetic variance for metrical traits in fish. Hybrid vigour is not uncommon, but appears frequently only in crosses of closely related species or subspecies. Heterosis is found in some racial crosses but more often in crosses of domesticated stocks where past history indicates that some inbreeding has taken place (Naevdal and Dalpadado, 1987; Krasznai, 1987).

Table 1.6 Type of sterility in fish hybrids (Chevassus, 1983)



1.2.4 Genetic manipulations in fish

In a normal fertilization, the spermatozoon triggers the resumption of the second meiotic division of the egg, which ends in the extrusion of the second polar body and the formation of a haploid female pronucleus. The fusion of the female and male pronuclei results in the first diploid nucleus of the embryo, which is then multiplied by a long series of mitoses (Chourrout, 1987). The techniques of artificial insemination developed make it possible to manipulate the sperm, eggs and zygote in a way that the genetic constitutions of the offspring and the populations are changed dramatically (Nagy, 1987). The main genetic manipulation techniques recently developed and used in different fish species are summarized in Figure 1.2.

Several methods which involve manipulation are preliminary to developing a broodstock for use in a breeding programme. Manipulations may alter productivity, fish quality and a mixture of natural physiological and genetic elements. The breeding approach is more purely genetic but is complicated, and that may affects practicability. These approaches seek to produce all male or all female fish through some forms of manipulation for each individual. Absolute success cannot be expected since individuals altered by the treatment are usually mixed in with unaffected fish in the same population.

Polyploidization

The production of polyploids can be achieved by manipulation of the early events following fertilisation. This usually means physical treatment (temperature shock or high pressure shock) of the zygote shortly after the fertilization to restore the second polar body (triploid induction) or shortly before the first mitotic division to cause endomitosis (tetraploid induction). Usually heat shocks are very effective for cold water fish (Chourrout and Quillet, 1982; Thorgaard *et al.*, 1983; Johnstone, 1985), but

these have also been successful applied to channel catfish (Bidwell, 1985) and tilapia (Chourrout and Itskovitch, 1983). The application of cold shock is more frequent for warm water fish, for tilapia (Valenti, 1975), carp (Gervai et al., 1980), channel catfish (Wolters *et al.*, 1982b), and African catfish (Richter *et al.*, 1987). However, in some species, heat shocks have become the preferred treatment because they require a shorter duration than cold shocks.

Beyond scientific interest, the polyploidization of fish has commercial applications too. One of the first expectations was a higher growth rate of the polyploids, since it was hoped that the increased gene complement might positively affect the phenotype, especially its growth hormone production. However, this expectation has not been fulfilled, at least in the species that have been examined. Wolters *et al.* (1982b) reported that the triploid channel catfish grew faster than normal diploids during the second season but no difference in the first season of growth in tanks. The accelerated growth of the triploids during the second season may be associated with lack of sexual development and not a direct effect of triploidy. Richter *et al.* (1987) studied the growth rate of the African catfish, and concluded that there was no significant effect of triploidy. However, Kowtal (1987), who studied white sturgeon reported that body composition was strongly affected. Triploid fish deposited less protein and more fat per unit weight than their diploid controls. No appreciable difference in growth was observed between triploids and diploids during the 10 weeks grow out period. As a general conclusion, in most cases, the growth rate differences, if there are any, may not become apparent in triploids until the onset of sexual maturation. Due to their sexual sterility and retarded gonad development, more energy can be utilized for growth during the normal period of maturation (Thorgaard, 1983; Scheerer and Thorgaard, 1989).

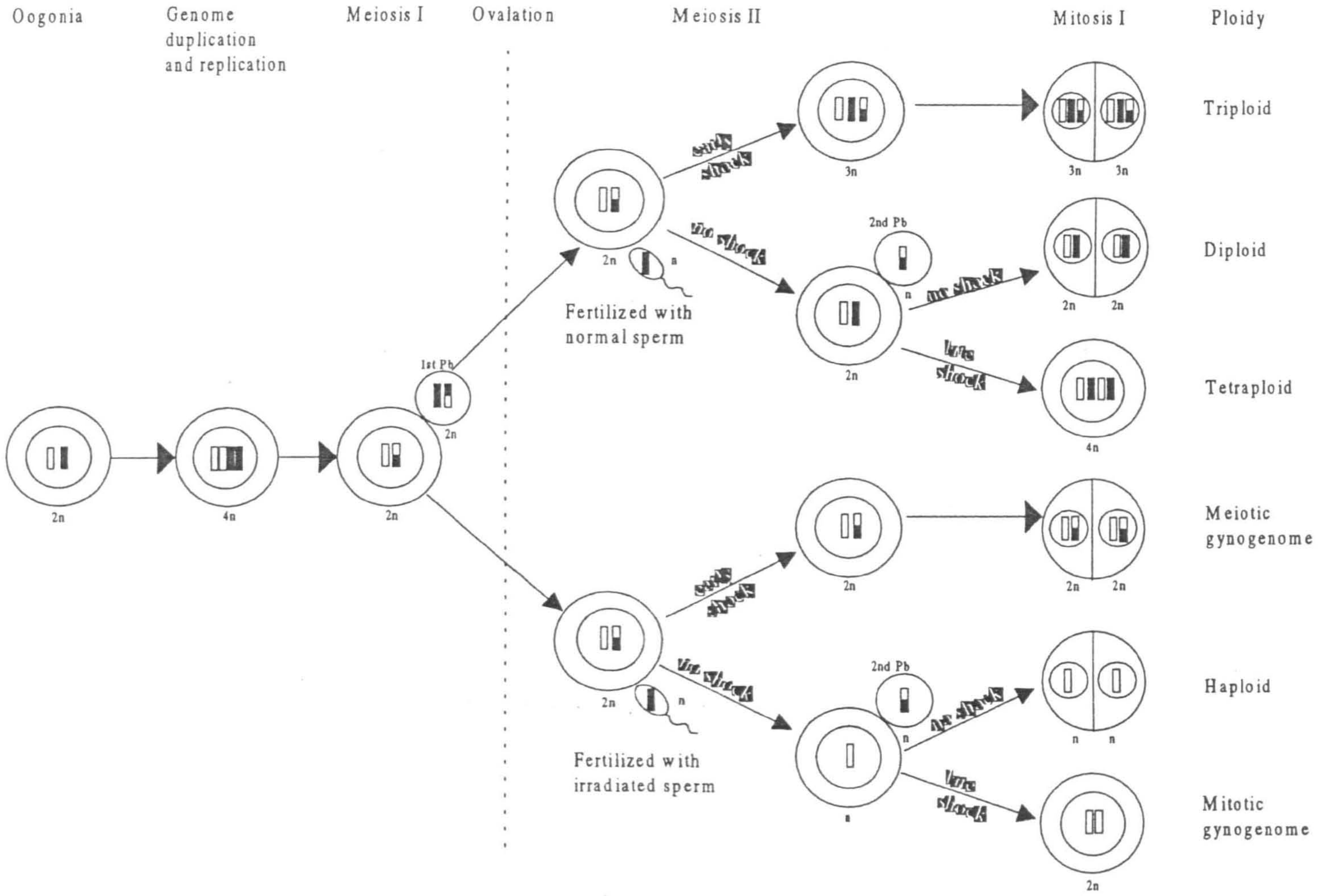


Figure 1.2 A schematic diagram of genetic manipulation techniques recently used in different fish species (modified from Hussain, 1992)

The sterility of triploids has a direct advantage in species with uncontrolled reproduction that cause difficulties in their culture. Gonad development of triploid males and females usually differs from that of their diploid fish. Usually, the gonad of a triploid male is less developed than that of the diploid, although the weight of both may not differ significantly. Spermiogenesis of triploids is disturbed by the irregular pairing of the three sets of chromosomes, and if spermatogonia develop, they are aneuploid (Thorgaard, 1983).

Manipulation of meiotic and mitotic events may produce polyploid individuals from ova that have been inseminated with genome-bearing sperm (Purdom, 1983; Thorgaard, 1983). The sperm status is the main distinction between induction of polyploids versus gynogenetics. The gynogenetic induction regime with reference to treatment, type and intensity of shock, time of application and duration can be readily optimized for a species by evaluating the yield of viable diploids, since the haploids die before swim-up. The analogous regime can be applied to polyploidization with anticipated optimal results simply by omitting the DNA-denaturing treatment of sperm. Evaluation becomes more complex since both diploid and polyploids are viable. Whether the sperm is from the same species as the ova donor or a related species will determine whether a polyploid species or a polyploid hybrid is produced, respectively. If an early shock is applied to increase the incidence of second polar body retention, triploid induction results. If shock is delayed until the period associated with the first cleavage, tetraploidy may result (Shelton, 1987)

Viability of intergeneric hybrids is a phenomenon that is frequently associated with polyploidy (Chevassus, 1983). Hybrids between remotely related species may result in sporadic production of viable offspring (Bakos *et al.*, 1978). Diploid hybrid viability may be low while triploids produced by retention of the polar body are more likely to survive, presumably because the complete genome from one parent species is only modified or influenced by the haploid set from the other parent species

(Chevassus *et al.*,1983; Scheerer and Thorgaard, 1989). The frequency of triploid hybrids should be increased by shock treatment as in gynogenesis. An application of this approach was reported with female grass carp, *Ctenopharhygodon idella* and male bighead carp, *Aristichthys nobillis* (Marian and Krasznai, 1978), to produce a sterile triploid hybrid (Krasznai *et al.*, 1984b). Contaminants included some viable diploid hybrids and female grass carp gynogens. These three phenotypes could be differentiated and culled (Cassani *et al.*, 1984; Krasznai *et al.*, 1984b), but the need to confirm ploidy led to improved techniques for separation. In addition to karyotyping, the larger nuclear size of polyploids permits differentiation based on red blood cell measurement, either microscopically (Allen and Stanley, 1978; Krasznai *et al.*, 1984a) or through use of a Coulter Counter (Benfey et al., 1984; Johnson *et al.*, 1984). Flow cytometry has also used to measure the greater DNA content in polyploid cells (Allen, 1983).

Subsequent testing of the triploid hybrid grass carp demonstrated the low weed control effectiveness, which reduced the attractivenesss of this sterile hybrid (Shireman *et al.*, 1983). The former successfully produced triploid grass carp, which further contributed to the demise of interest in triploid hybrid. The specific treatments to induce triploidy in grasss carp are largely the result of proprietary developments by commercial producers. Cassani and Caton (1986) have reported increased yield of triploids through various thermal shocks. Histological studies of gonads from triploid loach, *Misgurnus anguillicaudatus* (Suzuki *et al.*,1985), carp (Gervai *et al.*, 1980) and grass carp (Doroshov, 1986) have demonstrated probable sterility, however because of the differential gonadal development between the sexes, fertility tests should be applied to verify sterility.

Induction of triploidy has been reported for a variety of other fish, including rainbow trout (Chourrout, 1980; Lincoln and Scott, 1983), channel catfish (Wolters *et al.*, 1981), tilapias (Chourrout and Itskovich, 1983), Pacific salmonids (Utter *et al.*, 1983),

Atlantic salmon (Benfey and Sutterlin, 1984; Johnstone, 1985), European catfish, *Silurus glanis* (Krasznai *et al.*, 1984a), African catfish, *Clarias gariepinus* (Richter *et al.*,1987), Asian catfish *Clarias macrocephalus* (Vejaratpimol and Pewnim, 1990) and Asian catfish *Clarias batrachus* (Manickam, 1991).

Gynogenesis

Gynogenesis is the development of an ovum without a paternal genetic contribution (Thorgaard, 1983). The first application for gynogenesis is as a means of producing single-sex offspring, assuming the involvement of only the maternal genome which is of the homogametic sex. The genetic inactivation of the sperm is usually achieved by administering large doses of ionizing or UV radiation. After the radiation has completely destroyed the chromosomes, the spermatozoa retain the ability to penetrate into micropyle and activate the eggs and the cell division they initiate proceeds normally. Then, shocks to increase ploidy were applied shortly after fertilization, causing the retention of the second polar body. Thus, in the case of gynogenesis, it results in the union of two haploid complements produced in the second meiotic division. Late treatments are applied shortly before the expected time of first cleavage, and they therefore prevent spindle formation, resulting in endomitosis. The ploidy of the embryos is doubled after these shocks. It is easy to understand that completely homozygous diploid gynogenetic offspring can be produced by late shocks applied to haploid gynogenetic zygotes. Similarly, late shocks applied to normal diploid zygotes results in tetraploid offspring. A brief diagram of gynogenetic procedure is shown in Figure 1.3 (Chevassus, 1983; Purdom, 1983; Chourrout, 1987; Shelton, 1987).

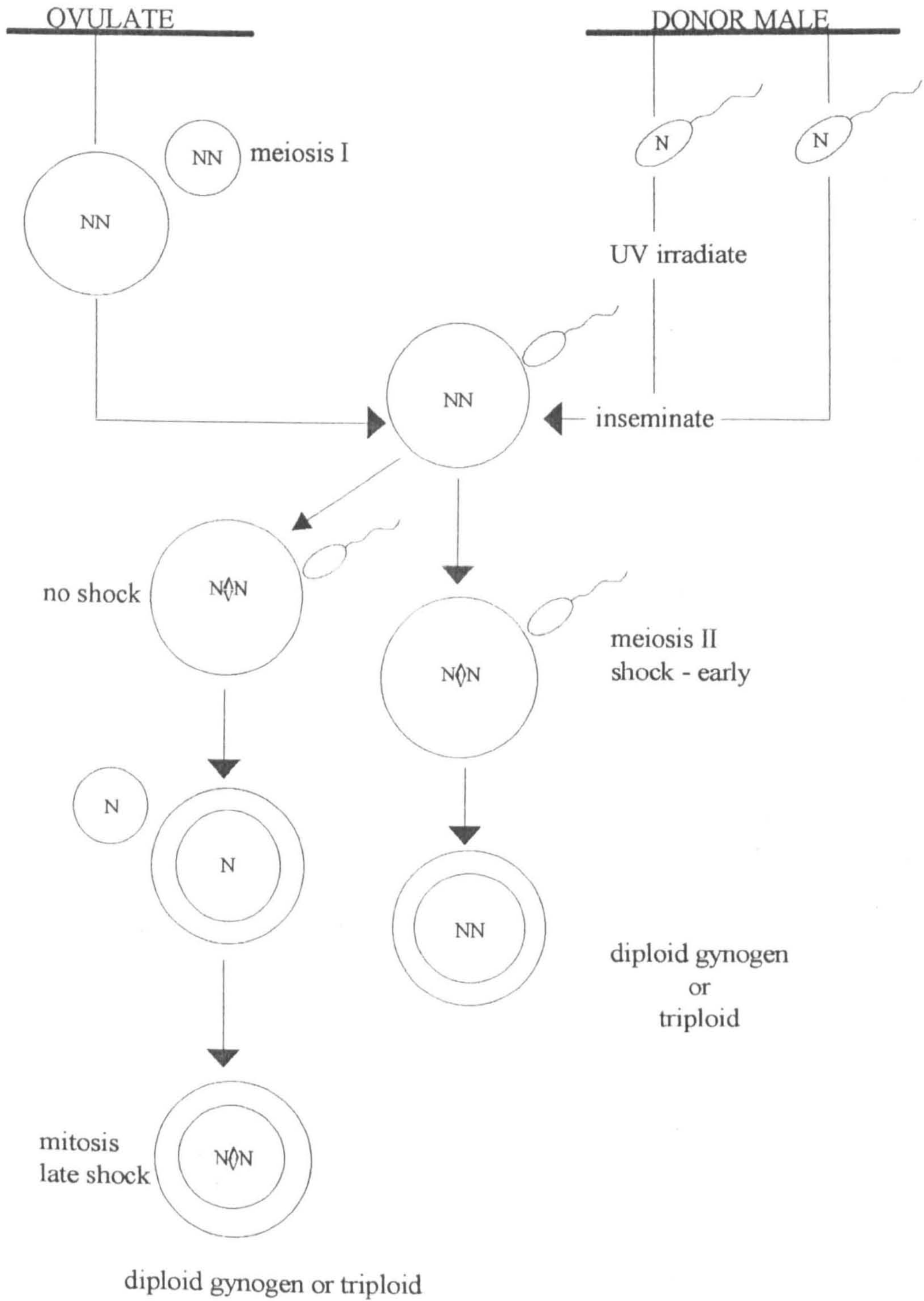


Figure 1.3 Chromosome manipulation to induce gynogenesis

In natural conditions, several fish species are represented by females only. One of the mechanisms of the reproduction of such species is gynogenesis, a kind of sexual parasitism. The oocyte of the female is fertilized by a male from a closely related species, but the genetic material of the sperm is eliminated during the early stage of embryo development (Nagy, 1987).

The various types of manipulation have been reviewed by Chourrout (1987). Most of them have been more or less precisely evaluated, particularly with regard to gynogenesis and polyploidization (Chevassus, 1987). The production of gynogenetics, androgenetics and polyploids has been described in several reviews (Cherfas, 1981; Chourrout, 1982; Thorgaard, 1983; Purdom, 1983; Chourrout,1987; Purdom, 1993 and Tave, 1993).

The artificial induction of gynogenesis is a very promising method for the aquatic sciences. Using this technique, the establishment of highly inbred lines can be achieved very quickly in aquatic species. These lines will surely improve fish culture by promoting the production of heterosis hybrids rather than selected lines (Tave, 1993).

The treatments used to produce diploid gynogenetic fishes have to overcome two problems: first, they must inactivate the genetic material of the sperm needed to initiate embryo development, and second, they must restore diploidy to the zygote, either by retention of the second polar body or by preventing the cellular division after the first mitosis. The solution of the first problem is based on the 'Hertwig effect'. The sperm, which were irradiated with ionizing or UV radiation in much higher doses than the lethal level, are used to initiate gynogenetic development. The restoration of the diploid stage is usually achieved by temperature or physical shocks to the zygote, as in triploidy induction (Nagy, 1987).

The variability of sex determination in fish is a critical consideration (Cherfas, 1981). Functional homogamety in fish has been demonstrated through gynogenesis in various cyprinids (Stanley, 1976b; Nagy *et al.*,1984; Siraj *et al.*, 1993; and Fujioka, 1993) and salmonids (Johnstone *et al.*,1978; Chourrout and Quillet, 1982; and Refstie *et al.*, 1982). Spontaneous gynogenesis through second polar body retention is rare, although the frequency can be increased by a variety of physical or chemical shocks at the appropriate time in relation to the second polar body formation. Chourrout and Itskovich (1983) and Chourrout (1984) demonstrated the value of optimization of various treatment parameters to increase the yield of heterozygous and homozygous diploid gynogenetics. Optimization has enhanced diploid production in some species, such as the plaice, *Pleuronectes platessa*; flounder, *Platichthys flesus* (Purdom and Lincoln, 1973); grass carp *Ctenopharyngodon idella* (Stanley, 1976a); common carp *Cyprinus carpio* (Nagy *et al.*, 1978); rainbow trout, *Salmon gairdneri* (Chourrout and Quillet, 1982); tilapias (Chourrout and Itskovich, 1983; Penman *et al.* 1987; Don and Avtalion, 1988b) and catfish *Clarias macrocephalus* (Na-Nakorn *et al.*, 1993). Some results were met to the levels that might be considered practical. Stanley (1979) produced enough female grass carp (*Ctenopharryngodon idella*) through gynogenesis for a large scale stocking test, but this was considered effort as diploid yield generally on the range of 0.1-10 %, principally without enhancement by shock. He also found little increase in yield with various treatments (Stanley, 1982). The females produced are fully fertile, and therefore, reproductive control through monosex stocking may be compromised if prior mixed-sex stocking has occurred. However, the main problem with many species is the low yield of diploids despite shock to enhance production (Nagy, 1987).

Induced gynogenetic diploidy can also be achieved through suppression of the first mitotic division in developing haploids. While this may be accomplished by judicious application of late shock (Thorgaard, 1983), the success is lower than in early shock. One aspect of optimizing gynogenetic treatments that has not been adequately

exploited is its value as an effective estimator of the most probable optimal treatment conditions for polyploidization. Shelton (1987) suggested that optimizing diploid production by either early (meiotic gynogenesis) or late shock (mitotic gynogenesis) should correlate with the greatest yield of polyploids: evaluation is direct through counting of surviving diploids in contrast to the necessity of ploidy evaluation when optimizing polyploidy directly.

1.2.5 Sex determination in fish

Fishes stand out in a special place among other vertebrates, as regards the gonosomal complement and sex determination mechanisms. First, among fishes, there is variety of sex chromosome types, from the undifferentiated sex chromosomes proper of most species of fishes to XY-XX; ZZ-ZW; XO-XX and multiple gonosome systems. Furthermore, these gonosomal types are found among many separate families and genuses, mostly without close phyletic relationships. Whenever there is a species having differentiated sex chromosomes, often there are other, closely related species displaying homomorphic chromosomes. Thus, the emergence of gonosomes seems to have occurred in many independent instances in fish. Second, fish show the only instances among vertebrates of natural hermaphroditism in a significant number of species, while separate sexes are present in the majority of teleostean species (Solari, 1994). Therefore, these characteristics, which show a large degree of plasticity in sex determinatio and in the occurrence of gonosome systems suggest that the study of sex determination in fishes may reveal important clues about the origin and function of sex chromosomes of other vertebrates.

The methods to control and manage fish reproduction, included: storing gametes; changing fish gender; manipulating the timing of reproduction and sterilizing fish are nescessary in this study, which concerns the ways to determine reproductive status and to manipulat various aspects of reproduction in catfish.

Dead fish can be sexes easily by direct examination of the gonads, however, indeveloping rapid and practical methods of sexing live fish would lead to improved estimates of stock reproductive potential, more efficient feeding and marketing of domesticated stocks, and more efficient sample sizes for sex specific experiments, among other advantages. live juvenile fish are especially difficult ti sex because they usually lack external features associated with sexual maturation, such as breeding tubercles or sexually dimorphic pigment patterns and urogenital pores (Crim and Glebe, 1994).

1.2.6 The histology of fish gonads

Efficient management of broodstock depends on an accurate prediction of ripening time to facilitate the collection of high quality eggs and sperm. From histological samples, ovarian tissues may be divided into eight stages of maturity (Marte and Lacanilao, 1986) based upon the dominant gametogenic cell types present: *Previtellogenic (immature) oocytes* are small, spherical ovarian cells containing a central nucleus and increasing amounts of cytoplasm (stage 1-3). *Vitellogenic (maturing) oocytes* (stage 3-6) incorporate the yolky materials produced by the liver. Yolk granules aggregate first at the periphery and later towards the center of the egg. *Mature oocytes* (stage 7) are the largest and are filled with yolk, which hardens during fixation and makes their histological preparation in paraffin difficult. The *spent ovary* (stage 8), found in females that have spawned, contains emtry follicles and postovulatory structures termed *corpora lutea*.

The developmental cycle of the fish testes can be divided into six stages based upon the appearance of the maturing germ cells. The cycle begins with spermatogonial cell proliferation (stage 1). Germ cell maturation proceeds with the appearance of spermatocytes (stage 2), spermatids (stage 3), and spermatozoa (stage 4). When males

are ripe (stage 5), termed the period of functional maturity, the testicular lumina are packed with sperm masses and milt flows freely when the abdomen is compressed. Stage 6, after spawning ceases, comprises a period of testicular involution and phagocytic absorption of residual sperm (Crim and Glebe, 1990).

1.3 Aims and structure of this thesis

The main aims of this study are concerned with the status of the pure species and the genetic manipulations of the hybrid catfish. This will involve:

1.3.1 **Electrophoretic study** of Thai clariid species to estimate variability and to determine the genetic relationships among the hybrid catfish and their parents.

1.3.2 **Karyotype analysis** of the parental and hybrid catfish.

1.3.3 **Triploidy induction** in hybrid catfish between female *Clarias macrocephalus* X male *C. gariepinus* and their reciprocal hybrids and its effect on survival rate, growth rate, gonad development and fertility.

1.3.4 Investigate sex determination using **gynogenesis**.

1.3.5 Look at the effects of using **different *Clarias gariepinus* strains in hybrid crosses.**

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Origin of fish.

The pure species (*Clarias macrocephalus* and *C. gariepinus*) used in this study were imported from the hatchery of the National Inland Fisheries Institute, (NIFI) Bangkok, Thailand to the Institute of Aquaculture, University of Stirling, United Kingdom in September-October 1991. Further generations were produced under laboratory conditions and maintained in the tropical aquarium of the Institute. The *C. macrocephalus* were originally wild stock from a paddy rice-field nearby Bangkok and had been kept at the NIFI hatchery for more than 8 years, while the African species was introduced to Thailand from Laos in 1987 (no other information is available on the origin of this stock).

2.2 Identification of Clariid catfish.

Identification of the species used the keys of Boulenger (1911), Smith (1945), Bell-Cross (1976) and Teugels (1986).

2.3 Fish stocks and their maintenance.

The newly imported catfish were placed in quarantine tanks and kept separately from other fish for at least 4 weeks. After the quarantine period, fish were transferred to stock tanks in recirculating systems. The system contained 2 x 180 litre head tanks plus 1 x 115 litre overflow tank, 4 x 180 litre plus 2 x 540 litre settling tanks with biofilters and 2 x 180 litre sump tanks attached to a 0.75 H.P. electric pump. There was a total of 16 fiber glass tanks (100 x 100 x 30 cm^3)

arranged in double rows of 8 tanks each on a two tier system. The tanks had valve controlled inlet pipes for incoming water and central standpipe venturi for discharging excess water and solid wastes (Figure 2.1). The flow rate of approximately 1 litre/ min/tank came directly from the header tank by gravity into each tank through 1 cm diameter inlet pipes and the waste water was discharged through a 25 mm stand pipe into a common settling tank and through a series of biofilter tanks into the sump tank before being pumped back to the header tank. All the tanks were kept at constant temperature of 28±1° C by a thermostatically controlled 3 KW heater (Howden Ltd.) placed in the header tank. Aeration was provided in each tank with one 15 cm length air stone linked to the central blower unit. Most of the tanks in this system were used for grow out fish as well as for all broodstock maintenance. Fish in all the above systems were maintained under 12 hour photoperiod, controlled by electric timers. Water qualities such as dissolved oxygen (5.0-7.0 mg/l), pH (6.5-7.8), ammonia (0-0.4 mg/l), nitrite (0.08-0.18 mg/l) and nitrate (10-20 mg/l) were regularily measured in all these systems.The fish were regularily monitored for diseases and parasites.

2.3.1 Egg incubation, fry rearing and on-growing system.

The egg incubation system for *Clarias* catfish was established in the tropical aquarium of the Institute of Aquaculture in 1992. The egg incubation system consisted of a 180 litre header tank, 1 x 180 litre and 1 x 540 litre settling tanks, a 0.25 H.P. pump and a unit of incubation tanks consisting of 12 x 20 litre plastic aquaria (Figure 2.2). The aquaria were connected to a recirculating system, where warm water was maintained at 28±1° C having a thermostatically controlled 3 KW heater (Howden Ltd.) placed in the header tank, the flow rate of water in each aquarium was approximately 1 litre/min. The water came directly from the header tank by gravity into each aquarium and the waste water was discharged into a common settling tank and through a biofilter tank before being pumped

back to the header tank, resulting a recirculating flow and removal of most of the waste products automatically.

The fertilised eggs were incubated at 28±1° C in 20 litre plastic aquaria in a recirculating water system as described above. Each plastic aquarium contained a small hapa with a piece of nylon mesh inside (0.5 mm mesh-size). The fertilised eggs were placed on this piece of nylon mesh : after hatching, the embryos passed through this mesh into the bottom of a hapa. The piece of nylon mesh containing egg chorions, dead embryos and unfertilised eggs was then removed from the incubation system.

The grow-out system for fry and fingerlings contained 2 x 540 litre header tanks plus 2 x 540 litre overflow tanks, 4 x 2,000 litre fibre glass settling tanks with biofilters and 2 x 180 litre sump tanks attached to a 0.75 H.P. pump (Beresford Pump Ltd.). There were 2 sets of circular rearing tanks connected with this water system, each set contained 32 x 20 litre circular plastic tanks arranged in double row 2 x 8 tanks each on a two tier sytem (Figure 2.3).

The advanced fingerlings rearing system consisted of 2 x 180 litre header tanks, 4 x 180 litre setting tanks, 2 x 180 litre sump tanks and a 0.75 H.P. pump and 2 sets of circular rearing tanks. Each sets consisted of 12 x 20 litre circular plastic tanks arraged on a two tier system which 6 tanks on each row (Figure 2.4)

2.3.2 Feeds and feeding.

The newly hatched fry can not swim up and settled at the bottom of the hapa until their yolk sac was resorbed, approximately 2 days after hatch. The early stages of fry were fed with *Artemia* nauplii for one week then changed to feed with wet trout pellet (No. 3 pellet). The fry were kept in the incubation system for at least

Figure 2.1 Glass fiber tank for broodstock rearing system.

Figure 2.2 Eggs and embryos incubator system.

Figure 2.3 Fry and fingerling rearing system.

Figure 2.4 Advanced fingerlings rearing system.

one week and then were transferred to the on-growing tanks or were sampled or disposed of if not needed.

All fish from fry to broodstock were fed commercial trout feeds (Ewos Baker Ltd. No.3 to No.5 pellet; 40-50 % crude protein, 15 % oil, 15 % carbohydrate, 10 % ash, 1 % fibre and 9 % moisture). The late fry received wet micronised No. 3 pellet which was blended to give a range of particle sizes (250-500 μm). The fry were fed initially at 25 % of body weight per day using the wet blended pellet, 3-4 times daily. The size of the pellet was increased to No. 3 as the fish grew after one month of age, they were given feed 2 times daily, at 5 -7 % body weight. The amount of the feed was reduced to 3-4 % body weight and the pellet size increased as the fish grew : No. 4 for 30 g up to 120 g and No. 5 for 120 g up to broodstock size. The fish were fed 7 days a week except on the periodical sampling days.

2.4 Anaesthesia.

In order to calm down or minimise handling stress, all fish were anaesthetized using ethyl 4-aminobenzoate (benzocaine) at a concentration of 100 ppm before all procedures which required handling fish (induced spawning, egg stripping, size measuring, tagging and blood sampling etc.). As benzocaine is not water soluble, a 10 % stock solution was first prepared in ethanol and required concentration was made by diluting the stock solution in water at the time of using. The fish were immersed in the diluted solution until their opercular movement ceased; in this condition they could be handled for up to 5 minutes. After carrying out the necessary sampling fish were moved to a bucket or aquarium with warm, clean water and strong aeration in order to aid their

recovery from anaesthesia and to avoid post- sampling mortalities. Generally, the fish recovered to normal condition within a few minutes.

2.5 Fish propagation, hormonal application, egg stripping and fertilisation

To prepare broodstock for propagation, it is necessary to keep the sexes separately to prevent the interruption from some feromones of the opposite sex which will be influence maturation circulate freely. The females were selected and pooled in one tank while special maintenance and feeding schedule was applied. Fish were fed with minimum 2 % body weight daily and kept with a good water flow and aeration for 2-3 months.

Sexually mature female catfish spawn at approximately 6-8 weeks intervals during the breeding season or under the experimental conditions described above : some mature females were implanted with synthetic LHRHa hormone (Hoechst, Germany) encapsulated in silastic tubing (Down Corning, USA). Readiness of females to spawn was ascertained by examining the degree of swelling of the belly and colouration of the urogenital papilla.The ripe females were collected and removed from the rearing tank to incubating tank. Spawning was induced by injecting the female with a mixture of LHRHa hormone and domperidone (Motilium-M:domperidone maleate; Janssen, Thailand). The dose applied was 20 µg of LHRHa plus 5 mg of domperidone per 1 kg of brooder weight. The brooders were kept individually in small incubating tanks which contained shallow static water. After a latency time of 15-18 hours, the eggs were manually stripped, by applying gentle downward pressure with the thumb and index fingers from just below the pectoral fins up to the genital opening of the fish. The eggs were collected in a clean, dry plastic bowl.

Immediately after stripping, the eggs were sub-divided into Petri dishes as the experimental design required. Milt from males can not be obtaining by stripping. The males have to be killed to get the testis for artificial fertilisation. Fertilisation of the eggs with the milt from macerated testes of the male fish was carried out later by using the semi-dry method (mixture of diluted sperm with modified Cortland's saline), followed by the addition of 10-20 ml of 28° C water. Eggs and sperm were mixed and stirred gently with a feather. Next, a little clean water was added and mixed two or three times to clean the fertilized eggs. After that fertilised eggs were left in the petri dishes for a few minutes before using for further treatments or transfer to the incubation system.

2.6 Collection, preservation and ultraviolet ray irradiation of sperm.

Milt was collected from macerated testes of the male fish. The sexually mature males were selected from broodstock tanks by examining the shape and coloration of the urogenital papilla. The male fish were killed, the testes was taken out, minced in a fine nylon net, then the milt were transfered to microcentrifuge tubes and centrifuged at 1,500 g for 10 min. at 4° C. The top clear supernatant was removed. Approximately 500- 1,000 µl of undiluted sperm was obtained in this way and kept separately for control in each experiment. Before any milt was used for normal fertilisation in hybridization (Chapter 3), triploidy (Chapter 4) or gynogenesis (Chapter 5), the motility of the sperm sample was always examined under a microscope. A small amount of milt (5-10 µl) was sampled and diluted with modified Cortland's saline (Appendix 1A) using an appropriate dilution factor depending on the initial concentration. The motility of the spermatozoa was checked by mixing 5 µl of diluted milt with 50 µl of water in a microcentrifuge tube and then rapidly placing a drop of the mixture on a glass slide for microscopic examination. The sperm motility in all samples was scored on a subjective rating scale system of 0 to 10. A rating of 10 denoted that 100 % of the

spermatozoa under observation were motile, moving actively, while 0 rating indicated that no sperm were moving after activation. Only diluted sperm samples with a motility score of 9-10 after activation were used. For short storage undiluted milt was held at 4° C in a refrigerator and found quite viable to fertilise eggs until 2-3 days after collection, while diluted milt with Modified Cortland's saline (approximately 1: 600) was viable to fertilise eggs until one week after collection.

Sperm concentration was estimated using a Neubauer counter (Haemacytometer, 0.1mm, 1/400 mm^2, Weber Scientific, England). Before the milt was used for any purpose, sperm head counts were made to estimate the whole sperm sample density. Dilution was done by taking 10 µl of sperm sample added in 490 µl of diluent (Modified Cortland's Saline), making a total of 500 µl sperm suspension in microcentrifuge tube. From the first dilution, 10 µl was drawn and added in 90 µl of diluent making a 1:50 and 1:10 dilution respectively. A small amount of sample was dropped on the Haemacytometer for counting (details as described in Appendix 1B).

Milt samples used for ultraviolet ray irradiation in all gynogenetic experiments described in Chapter 5 were first checked for motility and then sperm samples were diluted with a deactivator (Modified Cortland's Saline) to give cell concentrations of 2.5×10^7 or 2.5×10^8 ml^{-1} before irradiation with a 6 W ultraviolet lamp (wave length 254 nm) set using a radiometer (Ultra-Violet Products Inc., USA) to give a dose of 200 $\mu W\ cm^{-2}$ or 300 $\mu W\ cm^{-2}$ (Figure 2.5). Irradiation was carried out in a 4.5 cm diameter petri dish which contained 2 ml of diluted sperm and constantly agitated using an electric stirrer (Jencons miximatic, USA) at 4° C for durations as per design for each experiment.

2.7 Application of temperature and pressure shocks.

2.7.1 Application of temperature shocking.

Chapter 4 (induction of triploidy) and 5 (gynogenesis) describe the experiments involved in determining optimal shock treatments for ploidy manipulations. In this section, therefore, the apparatus and basic method are detailed. An 18 l water bath (temp. range 0-100° C capable of maintaining ±0.1° C) equipped with a heater, a cooler coil, a thermostatically controller and a water recirculating system (Julabo F18, Julabo Ltd.) was used for heat and cold shocking of fertilised eggs (Figure 2.6). About 30 minutes before the shock treatments were to be initiated, the water bath was filled with clean water and allowed to reach the required water temperature (typically between 2-5° C or 39-42° C). For extra accuracy, a mercury thermometer having 0.1° C division was used to finally adjust the temperature. Fertilised eggs to be treated were first placed in drip net(s) or piece(s) of nylon mesh floating on a bucket full of water (approximately 28° C) and then the drip net(s) or piece(s) of nylon mesh with fertilised eggs were shifted quickly into the water bath for the required duration as per design of the experiments. After the completion of temperature shock, drip nets or pieces of nylon mesh containing eggs were immediately moved back to the bucket full of water (approximately 28° C) and then directly transferred to the incubation system.

2.7.2 Application of hydrostatic pressure shocking.

The hydrostatic pressure shock treatments were used for optimising the inducing triploidy experiments described in Chapter 4. The machine used in these experiments had two vessels, which each contained 1.5 litre inside volume and was designed and built by Mr. Brian Howie, Chief Engineering Technician,

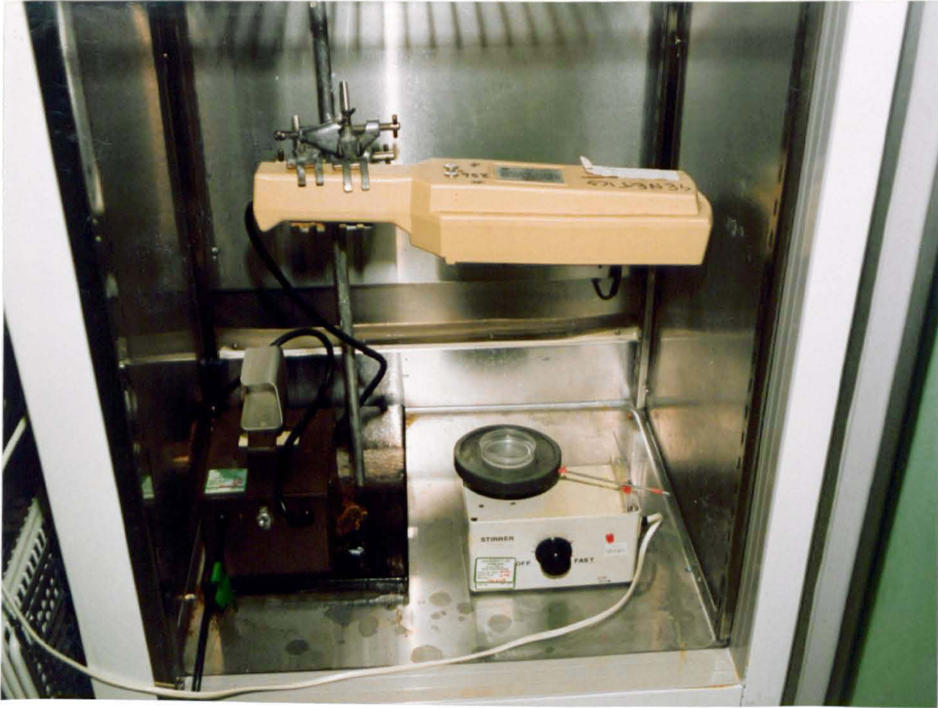


Figure 2.5 A 6 W (254 nm wave length) Ultraviolet lamp and an electric agitated stirrer setting in the refrigerator.

Figure 2.6 A thermostatically controlled 50 litre water bath for heat or cold shock treatment.

Institute of Aquaculture, University of Stirling, Stirling, Scotland (Figure 2.7). Before the shock treatments were to be initiated, the pressure vessel and hydraulic pump reservoir were first filled with clean water (temperature approximately 28° C). Fertilised eggs were placed in small pieces of nylon mesh which held on a bucket full of water (28° C) before being transferred into the vessels. After the vessels had been sealed and purged of air, the pressure release valve was closed and pressure was applied gradually by a manually operated hydraulic pump. The time taken to raise the pressure level from ambient to 8,000-9,000 p.s.i. was in the region of 30 sec. with the passage from 8,000 to 10,000 p.s.i. taking a futher 10 secs. Pressure was released by gradually opening the valve and the pressure dropped to ambient typically in 30 sec. After the pressure treatment, the pieces of nylon mesh containing the eggs were removed from the vessels and put back to the bucket full of water (approximately 28° C) before transfer to the incubation system.

Figure 2.7 A hydrostatic pressure machine which contained 2 x 1.5 litre vessels.

2.8 Criteria use for checking of embryonic development.

Fertilised treated and control eggs on the pieces of nylon mesh or drip nets were removed from the shocking equipment and floated in a bucket full of warm water (approximately 28° C). They were thoroughly cleaned and the number of eggs in each batch was estimated and recorded prior to incubation. After that the pieces of nylon mesh or drip nets containing the eggs were transferred and separately incubated in recirculating system as described above.

According to the previous results of microscopic examination of the development stage of fertilized eggs in *Clarias macrocephalus* as shown in Figure 2.8. The survival rate of embryos in each batch was checked at four development stages : fertilisation rate 6-8 hours after fertilisation (a.f.); somite stage 10-12 hours a.f.; hatching 22-24 hours a. f. and yolk sac resorption 3 days after hatch out, which were sampled three times in each batch of eggs and calculated as: (Average number of surviving embryos sampled at a given development stage/ average original number of sampled eggs) x 100.

2.9 Sampling, weighing and measuring

2.9.1 Weighing, and measuring fish

Fish sampling, weighing and measuring were essential procedures for data collection during experiments, periodical sampling and harvesting time in growth experiments (Chapter 3 and Chapter 4). Weighing of fish ≥2 g was carried out individually after anaesthetization (section 2.12) on a digital balance ranging 0.00-400.00 g (Metler PC 400, Metler Instrument AG). Early fry or fish ≤2 g were weighed wet in groups in a preweighed beaker containing water in order to

avoid handling mortalities. Measurements of standard length of the fish were carried out using Vernier callipers for small fish or a scaled ruler fitted on a wooden board for larger fish.

2.9.2 Fish tissue sampling.

Fish tissues for electrophoresis or histology were sampled from freshly killed or frozen fish using a scalpel, scissors and forceps. These tissues were frozen in a microcentrifuge tube at -25° C for electrophoresis or fixed in natural buffer (10% formalin) or Bouin's solution (Appendix 6) for histology until required. Special procedures for sampling skeletal muscle fins and barbels while the fish or broodstock were maintained alive for further experimental purposes were applied in this study, using biopsis. The fish were anaesthetized before all procedures. A small piece of caudal fin or barbels or a small piece of skeletal muscle were cut off using a sterile scalpel, scissors and forceps. After this a powder containing a mixture of an antibiotic and special glue (Orahesive protective powder, E.R. Squibb & Sons Ltd., England) was placed in the wound. The fish were tagged and moved to a 20 l plastic bucket with clean water and well aerated in order to aid their recovery from anaesthesia. After this, the fish were kept seperately in small tanks or glass aquaria until their wound recovered, then they were transferred to their former tank.

2.9.3 Fish blood sampling

Blood samples were drawn from the caudal vein of fish after anaesthetization (section 2.12) using a 25-gauge 2.54 cm hypodermic needle fitted to a 1 ml syringe using the same technique described in section 2.9.2. A few drops of blood were smeared and fixed on a glass slide for erythrocyte nuclear measurement or

the blood sample was allowed to clot and the serum and red blood cells were separately stored at -25° C until further use for starch gel electrophoresis.

2.10 Assessment of ploidy status.

2.10.1 Karyotyping

Karyotypes were prepared from newly hatched or 1 day old larvae from each batch by colchicine treatment and counting chromosomes according to the solid-tissue technique of Kligerman and Bloom (1977) and following modifications of published methods (Chourrout, 1982; Chourrout and Itskovich, 1983; Don and Avtalion, 1986; and Teugels *et al.*, 1992b). Embryos from each treatment group were placed in a 2.5 cm diameter petri dish containing 2-4 ml of freshly prepared colchicine (Sigma Ltd.) solution (approximate concentration 0.002-0.005 %) for 4-6 hrs. at 28±1° C. Body tissues were dissected from the embryos after transfer to chilled 0.7 % normal saline (NaCl) solution under a binocular microscope. The head, tail and yolk sac were removed using a pair of needles or fine forceps and the tissues were transferred into the distilled water (hypotonic solution) for 10-15 minutes. The tissues were then fixed in 4: 1 methanol- acetic acid, if tissues were to be stored then two further changes of fixative were given and the samples were kept in a refrigerator at 4° C for a maximum of 30 days.

For slide preparation, tissues were removed from the fixative, blotted to remove the excess fixative and then placed in the cavity of a perspex slide with 2-3 drops of 50 % glacial acetic acid (Analar Grade, Sigma Ltd.) and were minced for one minute with a glass rod or scraped by using fine forceps to dissociate epithelial cells. After 15- 20 minutes, 3-5 drops of cell suspension were dropped from 30-40 cm height on to a clean glass slide on a hot plate (45-50° C). The remaining liquid was then sucked back within 8-12 seconds into the micro-pipette dropper

(Drummond Scientific Co., USA.) leaving a fine and clean ring of cell spread. Slides were left for air drying for 30 minutes and stained with freshly prepared 10 % Giemsa stain (prepared by mixing Giemsa stain with 0.01 M phosphate buffer pH 7.0 ; Appendix 2A) for 20 minutes. Slides were rinsed in distilled water, air dried and mounted with DPX mountant (BDH Ltd.) after 10 minutes of xylene (BDH Ltd.) wash.

Metaphase spreads of chromosome were checked and chromosome numbers were scored by placing the slides under x400 and x1,000 (oil immersion) magnifications respectively using a compound microscope (Olympus Ltd.). Karyological examination was assessed by counting chromosomes of several (≥3) karyotypes per individual slide and 15-20 individuals per designed treatment group. Good metaphase chromosome spreads were photographed through a Leitz Orthomat photomicroscope (Leitz, Leitz & Wizard, Germany) under x1,000 (oil immersion) magnification. For establishing the karyotypes, the best photographs were used for cutting out, pairing and classifying chromosomes in increasing size, so that the karyotypes could be compared with each other.

2.10.2 Erythrocyte nuclear measurement.

Fish from each treatment group were anaesthetized (section 2.11) before collecting the blood samples. Two techniques were used to withdraw blood from the fish samples: 1) in the case of small fish (≤30 g body weight), the individuals were killed and the tail of each individual was severed behind the anal fin, a few drops of blood were placed at one end of a slide and smeared along the slide using the edge of another slide; 2) in the case of bigger fish, blood (0.1-0.2 ml) was sucked from the caudal vein of each fish using a 1 ml syringe and 25 G hypodermic needle. The needle was inserted at the position below the lateral line and level with behind the urogenital opening as to enter the vein just below the spine. The slides were prepared as mentioned before. Slides were air dried and

strained with Wright's blood stain (Appendix 2B) for 2 mins. and then dipped for 3 mins. in 1: 1 Wright's blood stain : Sorensen's buffer pH 6.7 (Appendix 2C). Slides were rinsed in distilled water, air dried and mounted with D.P.X. mountant (BDH Ltd.). Nuclear major axes were measured for ≥10 stained erythrocytes (RBC) on each slide (per individual fish) using an eye piece graticule at x1000 magnification using a compound microscope (Olympus Ltd.) as described by Penman *et al.*, (1987) or alternatively, fields of erythrocytes were photographed through a Leizt orthomat photomicroscope at x 1,000 on Ilford PAN F 50 film and developed using Kodak developer (Procedure described in Appendix 3). Negatives were projected with an enlarger to give a final magnification of x 3,000. This magnification enables an accuracy of ±0.1 μm. Major and minor axes of 20 erythrocytes from each fish were measured with calipers. The volume of each erythrocyte nucleus was calculated using the formula , volume = $4/3\ \pi\ ab^2$, where a was the major semi-axis and b the minor semi-axis of a perfect ellipsoid. The volume may have been slightly overestimated due to cell flattening. Frequency distributions plotted from these measurements and major axis/minor axis ratio were compared with the ploidy levels established from chromosome preparations. Mean major nuclear axis was selected for future experiments as being the only one of these variables for which the frequency distribution of diploid and triploid fish showed no overlap (Wolters *et al.*,1982a).

2.11 Starch gel electrophoresis.

The species of catfish, involved in this study were collected for the electrophoresis study (details are described in Chapter 3-5). Sixteen enzymes were surveyed and the procedure of electrophoresis is as given in the previous section. Types of alleles at the same locus were determined by the banding position of isozymes on the same gel and was based on the assumption that the isozyme bands migrating at the same position would have the same amino acid

composition and be coded by the same gene. Alleles were numbered consecutively as A, B, C,......or by relative mobility and so on, from the most anodal to the most cathodal side. Allelic frequencies were calculated by directly counting the phenotypes. Chi-square tests were used to examine the distribution of phenotypes in relation to their expected Hardy-Weinberg equilibrium distribution.

Starch gel electrophoresis (Sodsuk and McAndrew, 1991) was used as a technique to verify the genotype of various samples from broodstock, hybrid, triploid hybrid and gynogenetic diploid catfish. The general procedure of this technique is described as follow:-

2.11.1 Sample collection and preparation.

Tissue samples of the species of catfish were collected from fresh specimens. Small pieces of skeletal muscle, the entire liver and kidney, small pieces of caudal fin and barbel and the eye (without lense) from fresh killed or frozen fish were taken by scalpels, scissors and forceps. The number of individuals tested and sampling date were recorded. Identification of the species followed Smith (1945), Bell-Cross (1976) and Teugels (1986). Samples were obtained alive or kept in cool condition with dry ice during transportation, frozen and stored separately in microcentrifuge tube at -25° C in the laboratory until further use.

For electrophoresis, tissue samples were taken from cold storage thawed for a few minutes and then homogenized using a glass rod or plastic homogenizing rod placed into the microcentrifuge tube, then a small piece of Whatman No.3 filter paper (square cut in size 2 x 6 mm) was placed in the tube to absorb the crude homogenates of tissue sample onto the piece of paper.

For newly hatched embryos or early stages of fry, the samples were obtained alive and killed by refrigerating for 30 minutes. An individual fry was then placed in a microcentrifuge tube to which a few drops of homogenizing buffer (Aebersold *et al.*,1987; details in Appendix 4) were added, the embryos were homogenized using a plastic homogenizing rod fitted into the microcentrifuge, then a small piece of filter paper (square cut in size 2 x 6 mm) was placed in the tube, let the crude homogenates of hold fish sample to absorb onto a piece of paper for 15 minute. The samples were then loaded in the gel or kept at -25° C until further use.

2.11.2 Preparation of starch gel

About 23 g of hydrolised potato starch (Sigma Ltd.) was mixed with 220 ml of diluted TEB (22 ml buffer in 198 ml of distilled water) or CTC (8.8 ml buffer in 211.2 ml of distilled water) buffer (Appendix 5A) respectively in a Buchner flask. The mixture was heated with constant rotation of the flask to an almost translucent jelly state, quickly degassed using a vacum water pump and then poured into a 6 mm thick gel former. The gel, covered with a glass plate, was allowed to set at room temperature or left to cool down in the refrigerator at 4° C if required quickly.

2.11.3 Loading samples, runing, slicing and staining the gel

After setting hard, the gel was taken out of the former and a cut parallel to and 3 cm from the edge of the gel was made. The small pieces of filter paper which had absorbed crude homogenates of tissue samples were individually loaded along this cut with a maximum of 30 samples per gel. When all samples were correctly arranged the former was placed back on the gel and a perspex spacer (10 mm thick) was positioned between the gel and former to keep the sample slot closed.

The number and sequence of individuals loaded and sample species were recorded. After that the gel was placed in the electrophoretic bath with the appropriate buffer. A gauze wick soaked in the buffer was applied to either end of the gel to connect the gel and buffer solution. The bath tray covered with a transparent lid was placed in a refrigerator at 4° C, the power pack connected and set up with appropriate voltage and current and the gel allowed to run for 5-6 hours.

At the end of the run the gel was removed from the bath and loading sample papers were removed from the gel which was then sliced horizontally into three slices, each of which could be stained for a different enzyme system (Appendix 5). The appropriate stains (Appendix 5B) for the enzyme system to be examined were weighed and mixed with stain buffer solution and 2 % heated agar (approximately 50-60° C). The stain mixture was poured over the slice, allowed to set hard and then transfered to incubate at 40° C until the banding patterns became visible. The electropherograms were then analyzed and scored for the respective genotypes. When necessary for keeping records, the gels were preserved in gel fixative solution (Appendix 5C), dried and sealed between two layers of plastic or the top layer of agar was removed and fixed on a piece of filter paper, dried and kept sealed in plastic.

2.12 Sexing

In catfish broodstock and fish larger than 20 g (age over 3 months), sex was easily ascertained by examining the urogenital papilla. The urogenital papilla in male fish has a pointed shape, is comparatively longer than female and has a single common posterior opening; in the female fish it has a round-flat shape and shorter papilla with separate urinary and genital openings. Many hybrids and triploid fish with undifferentiated or poorly developed genitalia required

comparison on direct internal observation of gonads. If there was still doubt, the gonads were histologically examined to clarify identification of ovary and testis.

2.13 Histological procedure for gonadal tissue

Gonadosomatic Index and gonad histology , accurate assessment of reproductive maturity is most easily accomplished with dead fish. The relative gonad weight or gonadosomatic index (GSI) = 100x gonad weight(g) / body weight(g) is commonly used as a simple index of reproductive maturity. For microscopic analysis, whole gonads from dead fish, or gonad biopsies from live fish, will be preserved in formalin or other suitable fixatives and further processed according to standard histological techniques.

2.13.1 Gonadal collection and fixation

Fish gonads (testes or ovaries) were removed from freshly killed fish and immediately fixed in natural buffer (10 % formalin pH 7.6) or Bouin's solution (Appendix 6). The next day the gonadal tissues were rinsed and preserved in 70 % ethanol and stored in refrigerator at 4° C until further processong.

2.13.2 Tissue processing

The fixed gonadal tissues were cut into appropriate pieces (thickness≤4 mm), cassetted, labelled and autoprocessed on a Histokine tissue processor (Histokinette 2000, England). This involved passing tissues through different alcohol grades, followed by absolute alcohol, chloroform and then impregnation in molten wax. The processing procedure is described in Appendix 3.

2.13.3 Embedding

When the dehydration and infiltration of both testicular and ovarian tissues were completed, the tissues were embedded and blocked in a suitable size moulds using warmed paraffin (55-60° C) and cooled rapidly on a cold plate. After becoming hard the paraffin blocks were removed from moulds and stored at room temperature until sectioning.

2.13.4 Sectioning

The paraffin blocks were trimmed in order to bring the tissue to the surface. The blocks were then washed and cooled on a cold plate and tissues were sectioned to a thickness of 5 μm using a Leitz-Wetzlar microtome and Richert-Jung disposable microtome blades. Thin complete sections were floated on a warm water in water bath at 40° C and were collected on pre-washed glass slides. The slides were then labelled and dried overnight in an oven (at 60° C) before they were stained.

2.13.5 Slide staining.

The section slides were stained with haematoxylin and eosin (detailed in Appendix 3). Stained slides were washed with xylene for 5 mins. before mounting with DPX (BDH Ltd.). The permanent slides were stored in slide boxes for further examination and analysis.

2.14 Cruelty to Animals Act, 1876

The removal of blood samples and the administration of hormones, and colchicine were carried out under licence from the Home Office; they fell into the categories A (experiments performed without anaesthetics) and B (experiments performed under anaesthesia) as appropriate.

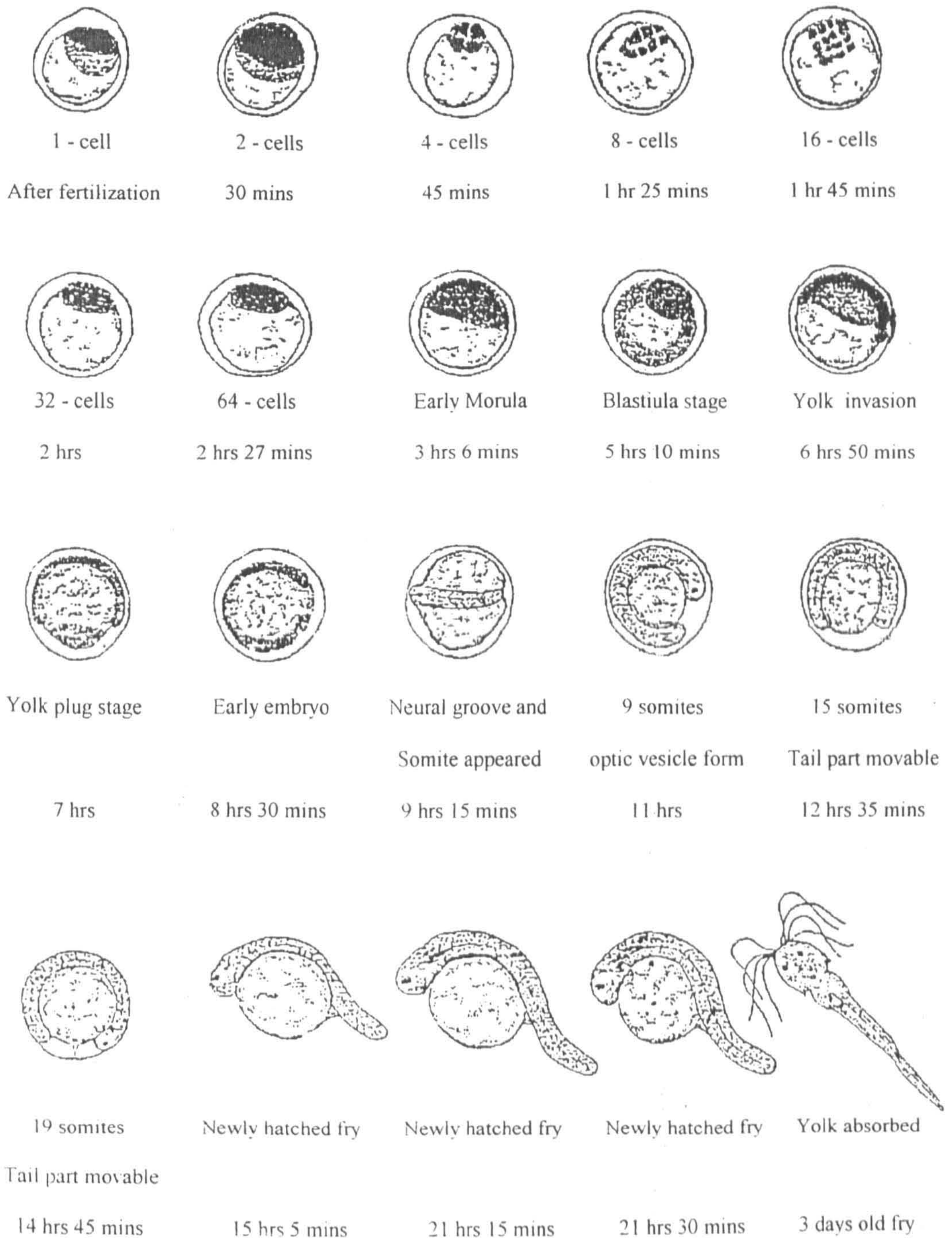


Figure 2.8 Development stage of fertilized eggs in *Clarias macrocephalus* 70x

CHAPTER THREE

ALLOZYMES STUDY IN *CLARIAS BATRACHUS*, *C. MACROCEPHALUS*, *C. GARIEPINUS* AND THE BIG-OUI HYBRID (FEMALE *C. MACROCEPHALUS* x MALE *C. GARIEPINUS*)

3.1 Introduction

Allelic and genotypic information obtained by electrophoretic methods permits genetic monitoring of hatchery and wild stocks. This important application of electrophoretic data has received relatively little attention to date in many countries. Monitoring can provide managers with critical genetic insights related to a population. Some uses of genetic monitoring with respect to hatchery practices have already developed in tilapiines, salmonids and cyprinides (Utter *et al.*, 1987, Whitmore, 1990). The collection and interpretation of genotypic data by electrophoresis is currently the primary means for measuring genetic variability within and among wild and cultured species of fish. It also has many practical applications within other genetic manipulation techniques such as comparisons between mitotic and meiotic gynogenomes (Hussain *et al.*, 1992).

Knowledge of taxonomy, accuracy in the identification of species and awareness of relationships among species are important for fish culture when attempting to introduce a new species, to produce large individuals and to control breeding lines. It is also necessary to investigate genetic characteristics of a population such as genetic variation in fish culture. Isozyme genes have been widely used as genetic markers to describe the genetic relationships among populations, subspecies, and closely related species (Whitmore, 1990; Utter *et al.*, 1987).

Nowadays, there is much information on genetic variation at the isozyme level in many fishes, but there are few investigations in *Clarias* catfish (Patimah *et al*, 1989; Teugels *et al.*, 1992). The nomenclature of the African clariid species has recently been revised by Teugels (1984). The African catfish, *Clarias gariepinus* (Burchell, 1822) and *Clarias lazera* (Cuvier and Valenciennes, 1840) are synonyms. Van der Walt, *et al.* (1993) selected four different *C. gariepinus* breeding pairs which each pair displaying different allele combinations (heterozygous) at the glucose-6-phosphate isomerase (*GPI-1* Or *2*) loci. They found that growth performance of the F_1 progeny of each breeding pair indicated the differences exist between different genotypes.

The objectives of the electrophoretic study described here were to estimate genetic variability within and between species and the inheritance of this variation using breeding experiments. This information will be used to identify species diagnostic alleles which will be essential to determine the specific status of broodstock and their purity, and the mode of inheritance in the hybrids and in genetic manipulation experiments.

3.2 Materials and methods

Samples of four groups of catfish namely the species *Clarias macrocephalus, C. batrachus, C. gariepinus*, and the big-oui hybrid (female *C. macrocephalus* x male *C. gariepinus*) were collected for the present study. The number of individuals tested and sampling dates are shown in table 3.1. Identification of the species followed Smith (1945), Boulenger (1911), Bell-Cross (1976) and Teugels (1986). Samples were obtained alive or kept cool with dry ice during transportation, frozen and stored at -20°C in laboratory. The cell-lysate obtained by freezing and thawing of these samples was then directly subjected to electrophoresis. A range of enzymes and proteins (following Patimah *et al.*, 1989 and Daud *et al.*, 1989) were surveyed

in liver, muscle and fin using starch gel electrophoresis. Electrophoresis was carried out in 12 % starch in appropriate buffer systems. A voltage of 40-60 mA (current) and 200 V (constant voltage) were applied for 5 hours at 4°C. The procedure of electrophoresis was as described in the previous chapter. The enzyme detection followed the methods used by Sodsuk and McAndrew (1991) in which the identification of the different alleles at the same locus was decided by the banding position of isozymes in the same gel. It was based on the assumption that the isozyme bands migrating to the same position were of the same amino acid composition and were coded by the same gene. Alleles were designated according to their mobilities relative to the most common allele in all species, which was designated 100; allelic variants were given numbers that indicate the mobility of their products relative to the common allele. Alleles were also numbered consecutively as A, B, C,....and so on, from the most anodal to the most cathodal side (Shaklee *et al.* 1990). Allele frequencies were estimated from genotypic frequencies by gene counting, since all protein variants observed in this study were interpreted to reflect products coded by codominant alleles. Allelic frequencies were calculated by directly counting the phenotypes. Chi-square tests were used to examine the distribution of phenotypes in relation to their expected Hardy-Weinberg equilibrium distribution. Expected heterozygosities (unbiased estimate of Nei, 1978) and the proportion of polymorphic loci were calculated for genetic variability measures in each species. Genetic differentiation was determined using the F-statistics of Wright (1978). The calculating formulae employed are shown in Appendix 5D. The software package BIOSYS-1 (Swofford & Selander, 1989) was used for calculation in this Chapter.

Table 3.1 Species, number, and date of collection of sampling specimens in this study.

Species (Abbreviation)	No. of fish	Date of sampling	Origin of fish stock
Clarias macrocephalus (Cm)	20	October 1991	Thailand, hatchery
Clarias batrachus (Cb)	20	September 1991	Thailand, hatchery
Clarias gariepinus (Cg)	20	September 1991	Thailand*, hatchery
Big-oui hybrid (Hyb)	20	April 1992	Artificial breeding

* imported from Africa to Thailand in 1987: no further information is available.

3.3 Results

Allozyme interpretation and description of enzyme banding patterns.

The electrophoretic conditions used in this study are given in Table 3.2. The 18 protein loci encoding the different enzyme systems were assessed from the comparison of the phenotypes observed in liver, muscle and fin and the variation among individuals in these three tissues. When no variation among individuals was observed within species, the enzyme was assumed to be encoded by only one locus in this tissue. The electrophoretic polymorphism observed in the 18 loci is shown in Table 3.3. The tissue specificity of the various enzymes did not appear to vary in any of the species studied.

ASPARTATE AMINOTRANSFERASE (AAT)

Two different loci, *AAT-1** and *AAT-2** were found. One of them, *AAT-2**, which appeared at the anodal zone, was not adequately resolved in all samples and therefore was not further taken into account. The products of *AAT-1** which was observed in the most of studied tissue appeared near the origin. *AAT-1** was monomorphic, a single invariant band in all the species studied.

ADENOSINE DEAMINASE (ADA)

A single *ADA** locus encoding for this monomeric enzyme was detected. This enzyme was observed in liver, muscle and fin tissue, but fin gave the stronger and clearer activity. Homozygotes were single-banded, heterozygotes double-banded. Polymorphisms with two different alleles of their parental species were found in big-oui hybrid. This enzyme system was a fixed heterozygote with two alleles expressed in the big-oui hybrid.

FUMARATE HYDRATASE (FH)

A single anodally migrating monomorphic locus was resolved for *FH**. This enzyme system was monomorphic in all species studied. The hybrids showed the interspecific allelic fixation of two different alleles between *C. macrocephalus* and *C. gariepinus*.

GLYCEROL-3-PHOSPHATE DEHYDROGENASE (G3PDH)

Only one locus, *G3PDH** was scored for this enzyme. The products of *G3PDH** were observed in liver, muscle and fin tissues. This enzyme system was fixed for alternate alleles in *C. macrocephalus* and *C. gariepinus*.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH)

A single *G6PDH** locus encoding for the dimeric enzyme was detected. This enzyme was observed in the three tissues studied and moved to the anodal zone. Monomorphism was detected in *C. batrachus* and *C. macrocephalus* with fixed interspecific mobility differences between *C. macrocephalus* and *C. gariepinus*.

GLUCOSE-6-PHOSPHATE ISOMERASE (GPI)

Two anodal loci, *GPI-1** and *GPI-2**, were scored for this enzyme in all species studied. The *GPI-1** products moved to the anodal zone and were usually observed near the origin, while the *GPI-2** migrated anodally. Both loci were observed in muscle tissue, whereas the *GPI-2** locus alone was observed in liver and fin in all species studied. Polymorphisms and fixed interspecific allelic mobility differences were observed in *GPI-2** loci. The three-banded heterozygotes, as well as heterodimeric hybrid bands between loci were observed (Fig. 3.1), indicating the dimeric structure of this enzyme molecule. Two GPI loci have been consistently recorded in African *Clariid* catfishes by Teugels *et al.* (1992).

ISOCITRATE DEHYDROGENASE (IDHP)

Two anodally migrating loci, *IDHP-1** and *IDHP-2** were observed in muscle and fin for *IDHP-1** and liver for *IDHP-2** respectively. *IDHP-1** was monomorphic in big-oui hybrid and their parental species. Polymorphisms as well as interspecific allelic differences between *C. macrocephalus* and *C. gariepinus* were observed at *IDHP-2**.

L-LACTATE DEHYDROGENASE (LDH)

Two loci, *LDH-1** and *LDH-2** were detected for this enzyme. These loci showed tissue specific activity. Muscle tissue was only active for *LDH-1** whereas liver and fin expressed *LDH-2** but *LDH-2** was the predominant locus in liver tissue. The products of both loci migrated anodally in all species. *LDH-1** was polymorphic and shared fixed interspecific allele mobility differences between *C. macrocephalus* and *C. gariepinus*. Five-banded heterozygotes, as well as heterotetrameric hybrid bands between loci were observed in the big-oui hybrid.

MALATE DEHYDROGENASE (MDH)

Two anodally migrating loci, *MDH-1** and *MDH-2** were observed. Tissue specific activity was found: *MDH-1** was predominantly active in liver and fin tissues, while *MDH-2** was found in muscle tissue. Single fixed monomorphic allele differences were observed between *C. macrocephalus* and *C. gariepinus* for *MDH-1**. *MDH-2** was polymorphic and shared fixed interspecific allele mobility differences between *C. macrocephalus* and *C. gariepinus*. Heterodimeric hybrid bands between loci were observed in the big-oui hybrid.

PHOSPHOGLUCONATE DEHYDROGENASE (PGDH)

A single anodally migrating *PGDH** locus was observed in liver, muscle and fin tissues in all species studied. Some mobility differences were observed between *C. gariepinus* and big-oui hybrid, while monomorphism and no interspecific mobility differences were found in both *C. batrachus* and *C. macrocephalus*.

PHOSPHOGLUCOMUTASE (PGM)

A single anodal *PGM** locus was observed in liver, muscle and fin tissues in all species. The structure of this enzyme could not be ascertained due to the expression of a single banded homozygotes with the same mobility between species. However, Teugels *et al.*, (1992) found a single *PGM** locus which showed double-banded heterozygotes in hybrids between *C. gariepinus* and *Heterobranchus longifilis*, suggesting that this enzyme is a monomer.

SUPEROXIDE DISMUTASE (SOD)

Activity reflecting only a single locus *SOD** was observed. This enzyme was detectable in liver, muscle and fin tissue and appeared in the anodal zone. A fixed difference was observed between *C. macrocephalus* and *C. gariepinus*, with three-banded heterozygotes, confirming the dimeric structure of this enzyme, present in liver tissue of the big-oui hybrid.

XANTHINE DEHYDROGENASE (XDH)

A single locus, *XDH**, was scored. This enzyme system which was observed in liver, muscle and fin in all species, appeared in the anodal zone. Only single-banded homozygotes were observed with the same mobility in all species.

XANTHINE-OXIDE DISMUTASE (XOD)

A single *XOD** locus encoding the dimeric enzyme was observed in liver, muscle and fin tissues of all species. Only single-banded homozygotes were observed which fixed for alternate allele between *C. batrachus* and *C. macrocephalus*.

Allozymic differences in species studied

Six loci, *ADA**, *GPI-2**, *IDHP-2**, *LDH-1**, *MDH-2** and *SOD** always clearly showed variation between the two parental species of big-oui hybrid. Two of these, *GPI-2** and *MDH-2** were found in later gels to be polymorphic in *C. gariepinus*, with two alleles (Figure 4.7;4.8 Chapter 4 and Figure 6.7 Chapter 6). The hybrids examined were heterozygous for these loci, with one of the *C. gariepinus* alleles and the single *C. macrocephalus* allele. No intraspecific variation was observed for *ADA**, *GPI-2**, *IDHP-2**, *LDH-1**, *MDH-2** and *SOD**: all hybrids showed the same heterozygote pattern for these loci. Figure 3.1 and Figure 3.2 shows the electrophoretic variation seen in *GPI-2**, *MDH-2** *LDH-1** and *ADA**. The hybrids also exhibited codominant alleles from *C. gariepinus* and *C. macrocephalus* at the other loci. These results confirm that interspecific fertilisation between both species resulted in hybrid progenies. No polymorphic loci were detected in the two Thai native species. The heterozygosity levels are shown in Table 3.3. It was found that the values in the two Thai native species were 0.000, *C. gariepinus* 0.078 and big-oui hybrid 0.667 respectively.

Genetic variability

Allele distribution and frequencies at 18 loci in the four groups of catfish are shown in Table 3.3. Genetic variability in the four groups was estimated by calculating heterozygosity from allele frequencies (Table 3.3). However, there were no polymorphic loci detected in the populations of *C. batrachus* and *C. macrocephalus*. Differences between *C. batrachus* and *C. macrocephalus* were observed at 6 discriminating loci.

The average number of alleles per locus was found to be highest in hybrids (1.7) and lowest in *C. batrachus* and *C. macrocephalus* (1.0). The proportion of polymorphic loci ranged from 0.000 in both *C. batrachus* and *C. macrocephalus* to 0.722 in hybrid. The observed average heterozygosity ranged from 0.000 in both *C.*

batrachus and *C. macrocephalus,* 0.078 in *C. gariepinus* and 0.667 in hybrid. The observed/expected average heterozygosity were 1.66 in *C.gariepinus* and 1.884 in hybrid. From these results, the big-oui hybrid catfish was found to be the most variable, followed by *C. gariepinus,* while the other two Thai native species showed less variability.

Some morphological characters of live and frozen specimens were examined during this study. The summary results are shown in Table 4.4 (Chapter 4). The hybrids have intermediate morphology between the parental species, corroborating the electrophoretic results.

3.4 Discussion

Genetic variability was estimated by calculating heterozygosity from the allele frequencies (Table 3.3). However, there were no polymorphic loci detected in the two Thai native species. For some loci, allele mobilities in *C. gariepinus* and big-oui hybrid did not appear to be identical. The big-oui hybrids came from a hatchery in Thailand where they were produced by pooled milt and eggs from different brooders during propagation. The hybrid samples did not came from a single pair of parents and the *C. gariepinus* used were not the actual parents of the hybrids.
The same conclusions resulted from a karyological analysis (in Chapter 4&5) of the species as studied in this chapter: *C. gariepinus* has the chromosome number of 2n=56, *C. macrocephalus* has 2n=54 and the big-oui hybrid has 2n=55, while *C. batrachus* was reported to have 2n=52 (Manickam, 1991). The cytogenetical data confirmed the electrophoretic results in this study. However, Teugels *et al* (1992) mentioned that it is a mistake to assume that a phenetic similarity in electrophoretic protein patterns or in karyotypes necessarily implies a close phyletic relationship or that these biochemical and cytogenetical data are some how more profound than morphological data. Using all three techniques should result in greater precision.

It was not appropriate to use the information on allele frequency to make assumptions about the populations of species used in this study because all of the fish had come from hatchery stocks, artificially maintained populations in which many of the assumptions underlying Hardy-Weinburg expectations would have been invalid. In this study, it was originally intended to obtain wild samples but those could not obtained due to a lack of time for collection.

However the main objectives of this electrophoretic study were to estimate genetic variability, within and between species and the inheritance of this variation using breeding experiments. The allozyme information from this study was used to identify species-diagnostic alleles, essential to determine the specific status of broodstock and offspring, and as markers for the mode of inheritance in the hybrids and genetically manipulated catfish described in the following Chapters.

Further electrophoresis work should be done, focussing on different wild populations in Thai native *Clarias* species. These and other allozyme techniques will be used to study the pure species broodstock and hybrids and develop techniques as markers in further genetic manipulation studies.

Table 3.2 Electrophoretic conditions used and genetic determination of the enzyme systems.

Enzyme	E.C. Number	Tissue	Locus	Buffer
Aspartate aminotransferase	2.6.1.1	L,M,F L	*AAT-1** *AAT-2**	TC
Adenosine deaminase	3.5.4.4	L,M,F	*ADA**	TC,TBE
Fumarate hydratase	4.2.1.2	L,M,F	*FH**	TC
Glycerol-3-phosphate dehydrogenase	1.1.1.8	L,M,F	*G3PDH**	TC
Glucose-6-phosphate dehydrogenase	1.1.1.49	L,M,F	*G6PDH**	TC
Glucose-6-phosphate isomerase	5.3.1.9	M L,M,F	*GPI-1** *GPI-2**	TC
Isocitrate dehydrogenase	1.1.1.42	M,F L	*IDHP-1** *IDHP-2**	TC
L-Lactate dehydrogenase	1.1.1.27	M L,F	*LDH-1** *LDH-2**	TC,TBE
Malate dehydrogenase	1.1.1.37	L,F M	*MDH-1** *MDH-2**	TC,TBE
Phosphogluconate dehydrogenase	1.1.1.49	L,M,F	*PGDH**	TC
Phosphoglucomutase	5.4.2.2	L,M,F	*PGM**	TC
Superoxide dismutase	1.15.1.1	L,M,F	*SOD**	TC,TBE
Xanthine dehydrogenase	1.2.1.37	L,M,F	*XDH**	TC
Xanthine-oxide dismutase	1.1.3.22	L,M,F	*XOD**	TC

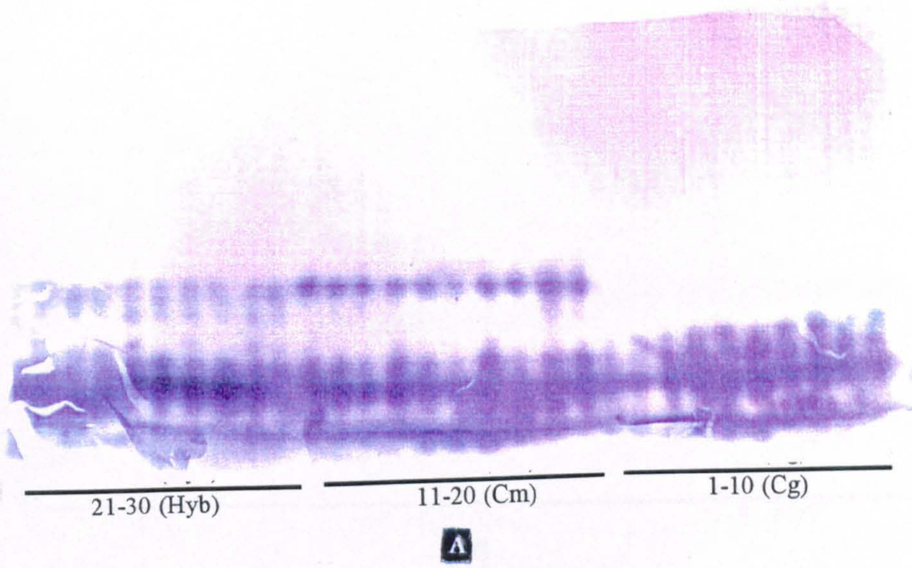
Table 3.3 Allele frequencies in the four species of *Clariid* catfish.

Locus	Allele	Species			
		Cb	Cm	Cg	Hyb
*AAT-1**	A	0	0	1.000	0.500
	B	1.000	1.000	0	0.500
*ADA**	A	0	0	1.000	0.500
	B	1.000	1.000	0	0.500
*FH**	A	0	0	1.000	0.500
	B	1.000	1.000	0	0.500
*G3PDH**	A	1.000	1.000	0	0.500
	B	0	0	1.000	0.500
*G6PDH**	A	1.000	1.000	0	0.500
	B	0	0	1.000	0.500
*GPI-1**	A	0	1.000	0	0.500
	B	1.000	0	1.000	0.500
*GPI-2**	A	0	1.000	0	0.500
	B	1.000	0	1.000	0.500
*IDHP-1**	A	0	1.000	1.000	1.000
	B	1.000	0	0	0
*IDHP-2**	A	1.000	1.000	0	0.650
	B	0	0	1.000	0.350
*LDH-1**	A	1.000	1.000	0	0.500
	B	0	0	1.000	0.500
*LDH-2**	A	1.000	0	0	0
	B	0	1.000	1.000	1.000

Table 3.3 (cont'd)

Locus	Allele	Species			
		Cb	Cm	Cg	Hyb
*MDH-1**	A	0	0	1.000	0.500
	B	1.000	1.000	0	0.500
*MDH-2**	A	1.000	0	1.000	0.500
	B	0	1.000	0	0.500
*PGDH**	A	1.000	1.000	0.800	0.850
	B	0	0	0.200	0.150
*PGM**	A	1.000	1.000	1.000	1.000
*SOD**	A	0	0	1.000	0.500
	B	1.000	1.000	0	0.500
*XDH**	A	1.000	1.000	1.000	1.000
*XOD**	A	0	1.000	1.000	1.000
	B	1.000	0	0	0
Average No.of allele		1.0	1.0	1.1	1.7
Proportion of polymorphic loci		0	0	0.111	0.722
Observed average heterozygosity		0.000	0.000	0.078	0.667
Expected average heterozygosity		0.000	0.000	0.047	0.354
Observed / Expected		1.000	1.000	1.660	1.884

Abbreviations (Cb, Cm, Cg and Hyb) as used in Table 3.1.



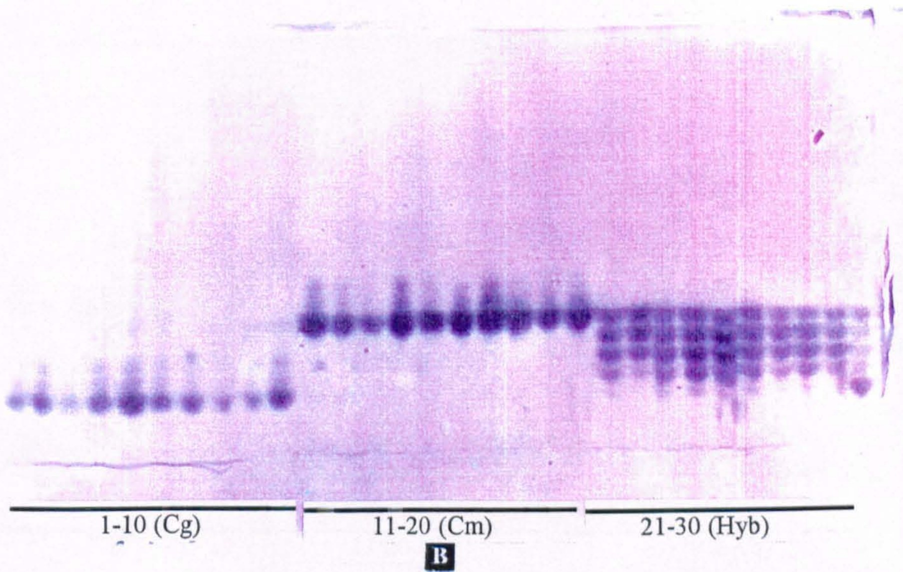


Figure 3.1 Some electrophoretic variatios observed from muscle tissue in 10 each of *C. gariepinus*, *C. macrocephalus* and Big-oui hybrid in:

(A) *GPI-2 (start from right hand side)**

(B) *LDH-1 (start from left hand side)**

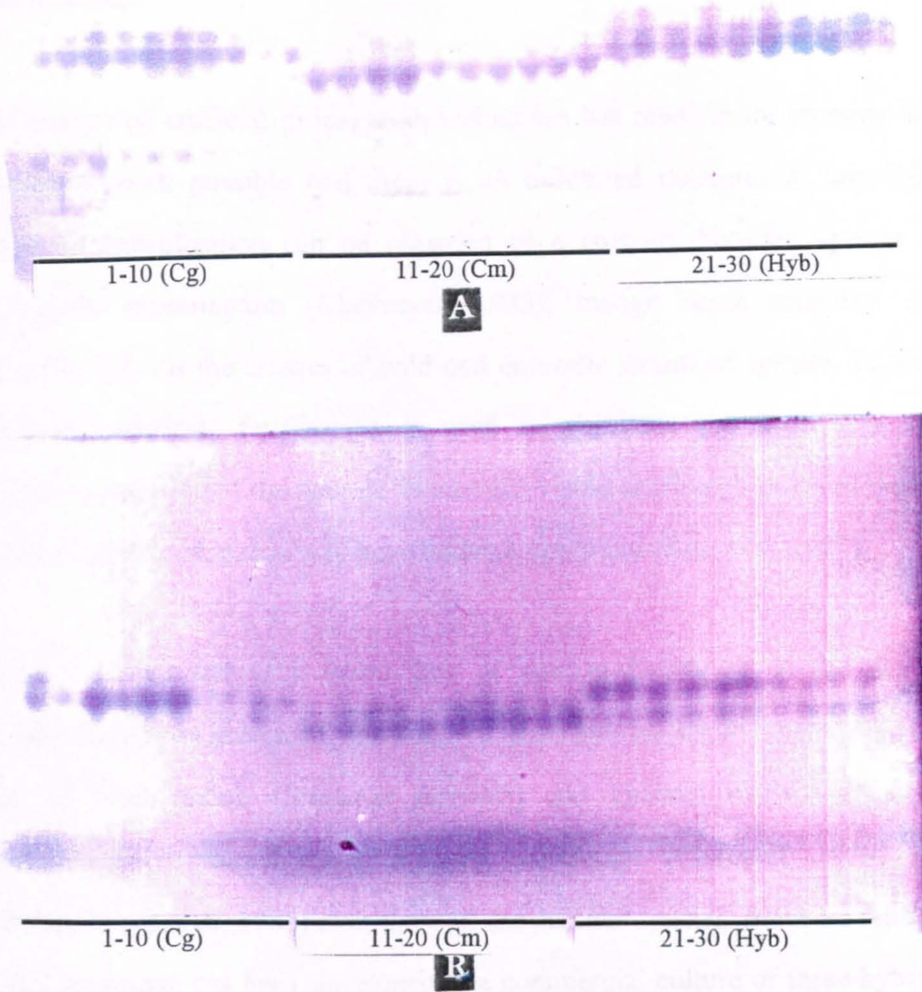


Figure 3.2 Some electrophoretic variatios observed from muscle tissue in 10 each of *C. gariepinus*, *C. macrocephalus* and Big-oui hybrid in:

(A) *MDH-2 (start from left hand side)**

(B) *ADA (start from left hand side)**

CHAPTER FOUR

HYBRIDIZATION STUDIES IN *CLARIAS MACROCEPHALUS* AND *C. GARIEPINUS*

4.1 Introduction

The advantage of artificial propagation techniques has made more crossing and hybridization work possible and there is an unlimited potential in this field. Interspecific hybridization can be regarded as a crossing between species or heterospecific insemination (Chevassus, 1983), though some scientists call interspecific hybrids the crosses of wild and domestic strains of species. In most experiments, artificial fertilisation is used to eliminate the ecological and ecological segregation of the species. Numerous viable artificial hybrids of warm-water fishes produced in this way have become important (Krasznai, 1987).

In catfish, several hybrid combinations of species belonging to the family *Ictaluridae* have been produced, and some of these hybrids showed heterosis. The hybrids of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) have increased yield, dressing percentage (Chappell, 1979) and catchability (Tave *et al.*,1981) over those of the parental species. Since no reliable spawning technique has been developed, the commercial culture of these hybrids has not begun. In *Clarias* catfish, artificial hybridization between two African catfish, *Clarias gariepinus* and *Heterobranchus longifilis* was performed. The hybrids revealed an intermediate karyotype and it appears as if they have totalized a haploid chromosome set from both parental species. The hybrid karyotype is considered as aneuploid, although the hybrids proved to be fertile (Teugels *et al.*,1992).

The hybrid between female *Clarias macrocephalus* and male *C. gariepinus* has become an increasingly important fish for aquaculture in Thailand and some neighbouring countries. Since the first successful artificial hybridzation in 1988, no detailed analysis of the genetic characteritics of this hybrid have been undertaken. The present investigation has been carried out aimed at improving the efficiency of production and to gain an understanding of the genetic status in this hybrid catfish. This has included comparisions of fertilisation and hatching rates and survival to yolk-sac resorption in the hybrid, its reciprocal hybrid and the parental pure species. Genetic studies included, karyotyping of each species, electrophoretic studies to estimate genetic variability and to determine the genetic relationships between the hybrid and the parents and morphological characteristics of the pure species and the hybrid.

4.2 Materials and methods

Pure species (*C. macrocephalus*, *C. gariepinus*) and the hybrid (female *C. macrocephalus* x male *C. gariepinus*) were imported from the hatchery of the National Inland Fisheries Institute, Thailand in 1991-1992 and reared in the tropical aquarium of the Institute of Aquaculture, University of Stirling, under simulated normal photoperiod (12D:12L) and ambient temperature (28° C). The African species *C. gariepinus* was introduced to Thailand from Laos: no other information is available on the origin of this stock. Another two stocks of *C. gariepinus* which were used in this studied were imported to the Institute of Aquaculture in 1985, one from Malawi and another from Wageningen, the Netherlands. Morphological identification of the species used the keys produced by Boulenger (1911), Smith (1945), Bell-Cross (1976) and Teugels (1986).

Hybrid crosses were produced under laboratory conditions by injecting the female of either species with LHRHa hormone: after a latency time of 15-18 hours, the eggs

were hand-stripped and fertilized with milt from the macerated testes of a mature male. The fertilized eggs were incubated at 28° C in 20 L plastic aquaria in a recirculating water system. Each plastic aquarium contained a small hapa, within which the eggs were placed on a piece of nylon mesh (0.5 mm mesh-size) to assist in the removal of egg debris after hatching. At about 22-26 hours after fertilization, hatching takes place. Fertilizations, hatching rates and survival rates were compared among the hybrid, reciprocal crosses, and parental pure species.

The back cross hybrids were studied using the big-oui hybrid (F_1) stock produced during the first year of this study in 1992. The F_1 hybrids were crossed back to their parental species, both *C. macrocephalus* and *C. gariepinus.* Fertilizations, hatching rates and survival rates were compared in the back cross hybrids relative to the big-oui hybrid.

The investigation on effects of using different *C. gariepinus* male broodstocks in hybrid crosses were carried out following the same procedure as above, by using the sperm from another two stocks of *C. gariepinus* (Malawi and the Department of Fish Culture and Fisheries, Wageningen Agricultural University, Wageningen, the Netherlands). Fertilisation rates, hatching rates and survival rates were compared with the hybrid crosses producing from male broodstocks from Thailand.

About 20 newly hatched larvae from each batch were sampled and karyotyped according to the solid-tissue technique of Kligerman and Bloom (1977). Metaphase chromosome spreads were photographed through a photo microscope under x1,000 magnification (oil immersion). The clearest photographs were used for classifying chromosomes and karyotypes (details in Chapter 2).

Samples for allozyme studies were collected from freshly killed fish tissue samples (muscle, livers and fins), frozen and stored at -20°C until tested. Nineteen enzymes

were surveyed using starch gel electrophoresis. Electrophoresis was carried out with 12 % starch in a continuous buffer system of Tris-citrate pH 8 (TC) or Tris-borate-EDTA pH 8.5 (TBE), both at a voltage of 40-60 mA and 200 V (constant voltage) for 5 hours at 4°C (for details see section 2.10, Chapter 2). The enzyme detection followed the methods used by Sodsuk and McAndrew (1991) and Teugels *et al.*, (1992b). The nomenclature and allele designation followed that of Shaklee *et al.*, (1990).

4.3 Results

4.3.1 Fertilisation and survival of big-oui hybrid, reciprocal cross hybrid and their parental species

The comparisions of fertilisation rate, survival to somite stage, hatch out and yolk-sac resorption from 3 replicate experiments are shown in table 4.1. The big-oui hybrid had fertilisation rates and survival rates at the various developmental stages which were similar to those of the pure species. The survival in the reciprocal hybrid was always significantly lower at every stage of larval development: the means with standard error of survival of embryos are shown in table 4.1.

4.3.2 Back cross hybrid

The comparisions of fertilisation rates and survival rates at various development stages from 3 replicate experiments are shown in Table 4.2. All back cross hybrids and F_2 hybrids were always significantly lower at every stage of larval development than the big-oui hybrid. Most back crosses produced very poor viability in embryos with a small number of deformed hatchings which died soon after hatching. Only the crosses between female F_1 hybrid and male *C. gariepinus* produced vilable embryos

and fry, giving 8.00±0.42 % survival to yolk sac resorption relative to normal big-oui hybrid.

A few of these backcross hybrid fry were reared for more than one year but did not reach maturity. Most of these fish have abnormal body shape (Figure 4.1), more aggressive behaviour than the big-oui hybrid and tolerance to environmental changes.

The cross between female F_1 hybrid and male F_1 hybrid (F_2 hybrid) never developed to hatching. The fertilized eggs developed to somite stage, then suddenly stopped developing and died at this stage.

4.3.4 Karyotyping

Karyotyping of pure *C. macrocephalus* showed a modal chromosome number of 2n=54, *C. gariepinus* showed a number of 2n=56 and the big-oui hybrid had a modal chromosome number of 2n=55, as results were described by Lawonyawut *et al.* (1993b). The reciprocal hybrid karyotype of 2n=55 indicated that the hybrid receives a single haploid complement from each parent species (table 4.3 and figure 4.2-4.6). The back cross hybrid also had a modal count of 2n=55 chromosomes.

Table 4.1. Mean survival rate of big-oui hybrids, reciprocal crosses hybrids and the parental pure species at different development stages.

Pair-crosses female X male	Expected Larvae	% ± SE			
		Fertilisation rate	Somite stage	Hatch out	3 days of age
Cg X Cg	*Clarias gariepinus*	83.59±2.87[a]	75.08±4.59[a]	56.04±22.33[a]	37.89±4.59[a]
Cm X Cg	Big-oui hybrid	85.14±4.59[a]	73.59±12.12[a]	63.27±16.26[a]	42.48±2.29[a]
Cg X Cm	Reciprocal hybrid	30.78±1.72[b]	14.18±9.21[b]	3.29±2.29[b]	0.01±0.57[b]
Cm X Cm	*Clarias macrocephalus*	80.60±1.72[a]	68.25±9.79[a]	54.91±5.16[a]	41.69±4.59[a]
± S.E.M.	-	±2.73	±8.93	±11.51	±3.01

Superscripts denote significant differences between values by ANOVA at level $p < 0.05$

Abbreviation; Cg = *C. gariepinus*, Cm = *C. macrocephalus*

Table 4.2 Mean survival rate (relative to control; big-oui hybrid) of back crossing hybrid and F_2 hybrid.

Crosses	mean survival % ± S.E.			
female x male	Fertilisation	Somite stage	Hatch out	3 days of age
Cm x Cg	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Hyb x Cg	94.48 ± 0.33^{b}	86.44 ± 0.64^{b}	16.66 ± 0.27^{b}	8.00 ± 0.42^{b}
Hyb x Cm	54.43 ± 1.36^{c}	27.93 ± 0.46^{c}	3.09 ± 0.23^{c}	0.00 ± 0.00^{c}
Cg x Hyb	52.13 ± 0.58^{c}	12.19 ± 0.16^{d}	1.94 ± 0.57^{cd}	0.00 ± 0.00^{c}
Cm x Hyb	53.82 ± 0.52^{c}	24.24 ± 0.12^{c}	0.07 ± 0.02^{de}	0.00 ± 0.00^{c}
Hyb x Hyb	25.66 ± 0.06^{d}	11.78 ± 0.18^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
±SEM	0.48	0.26	0.18	0.07

Data in the same column carrying different superscripts denote significant difference by ANOVA between values in each development stage at 95 % confident level.

Abbreviation,

Cg: *C. gariepinus*

Cm: *C. macrocephalus*

Hyb: big-oui hybrid

Figure 4.1 The back cross hybrid (female F_1 big-oui hybrid X male *C. gariepinus*), showing abnormal body shape.

Table 4.3. Frequency distribution of diploid chromosome numbers in big-oui hybrid, reciprocal hybrid, back cross hybrid and their parental species.

Crosses	No. of	Chromosome numbers								No.of	Modal
pair FxM	larvae	50	51	52	53	54	55	56	57	spreads	count
CgXCg	20	1	0	3	4	10	14	41	3	76	56
CmXCg	20	5	8	16	27	28	66	3	0	142	55
CgXCm	20	2	0	5	3	10	18	0	0	38	55
HybXCg	20	5	3	11	9	15	53	0	0	96	55
CmXCm	20	0	6	19	24	59	4	1	0	113	54

Abbreviation; Cg = C. gariepinus, Cm = C.macrocephalus, Hyb = big-oui hybrid.

4.3.4 Maturity and gonad development

The big-oui hybrids had a 50 : 50 sex ratio and both sexes developed gonads. In the males, the testes were reduced compared to the pure species but contained some spermatozoa which were incapable of fertilizing either parental species. In the female hybrids usually only one of the ovaries developed, but the eggs matured and could be succesfully fertilized by *C. gariepinus.* Crosses between male and female big-oui were unsuccesful as was also noted by Nukwan *et al* (1990).

4.3.5 Morphometry and electrophoretic studies

The allozyme study analysed 18 different loci, of which 3 (*GPI-2**, *LDH-1** and *MDH-2**) were clearily diagnostic for the two parental species (fixed allele differences). The big-oui hybrids were always heterozygous for these (Figure 4.7) as were the few surviving reciprocal hybrids that could be analysed.

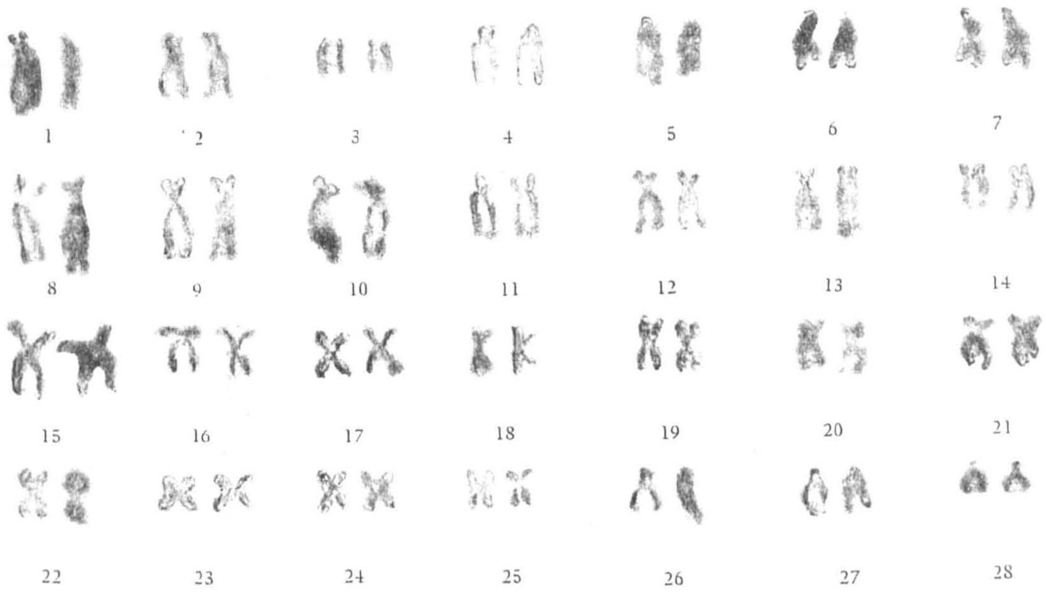


Figure 4.2 Representative karyotype of *C. gariepinus*, 2n = 56

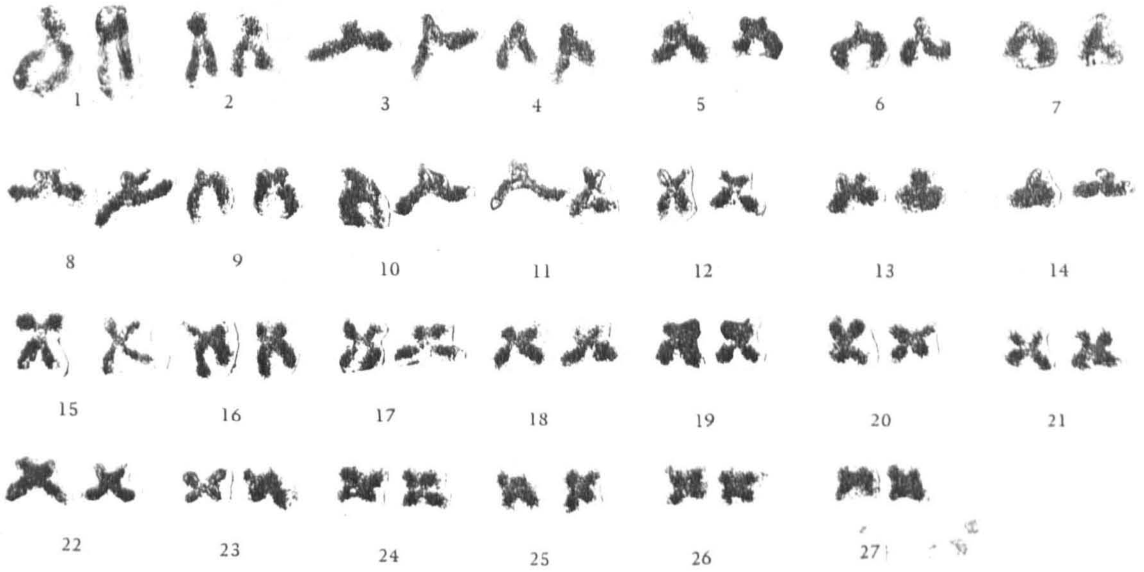


Figure 4.3 Representative karyotype of *C. macrocephalus*, 2n = 54

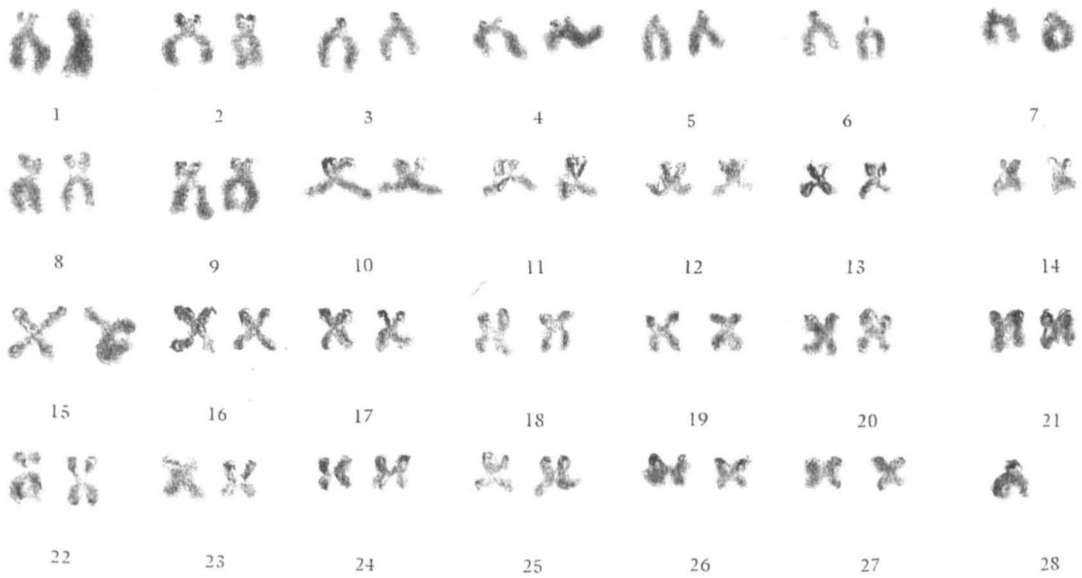


Figure 4.4 Representative karyotype of big-oui hybrid (female *C.macrocephalus* X male *C. gariepinus*), 2n = 55

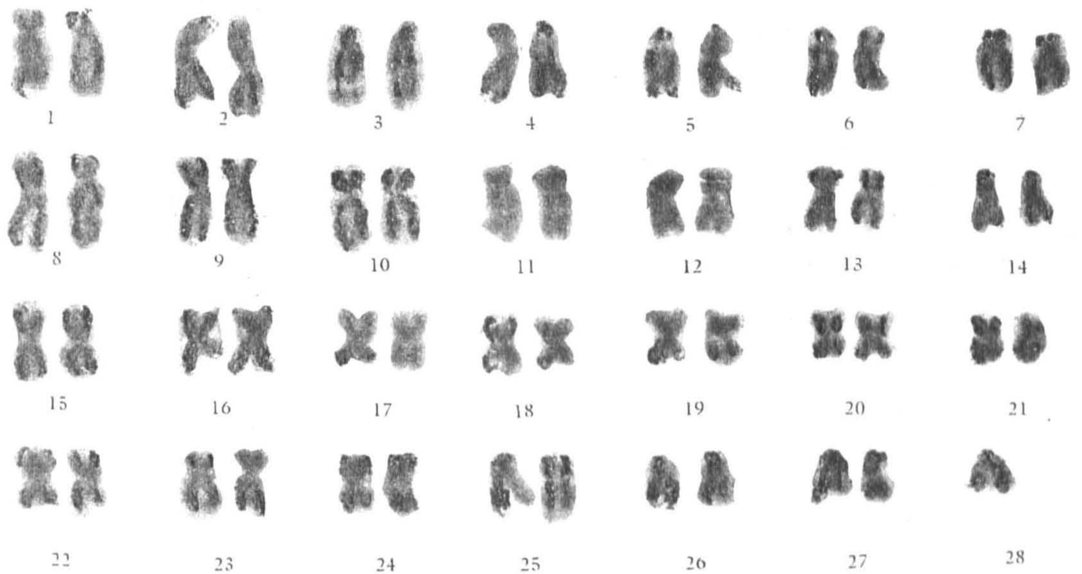


Figure 4.5 Representative karyotype of reciprocal cross hybrid (female *C.gariepinus* X male *C.macrocephalus*), 2n = 55

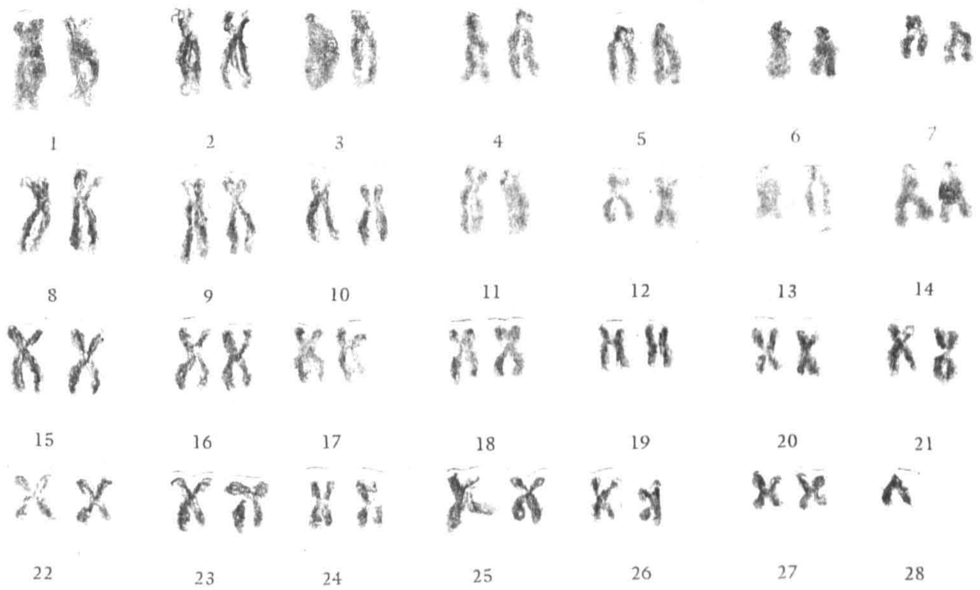


Figure 4.6 Representative karyotype of back cross hybrid (female F_1 big-oui hybrid X male *C. gariepinus*), 2n =55

The back cross hybrids in Figure 4.8 (9 individuals) were all heterozygous for these three loci, similar to the F_1 big-oui hybrids. In total, the back cross hybrid tested (29 individuals: 20 newly hatch fry and 9 adult fish) were all heterozygotes and significantly different from the expected ratio of 1:1 heterozygotes: homozygotes (*C. gariepinus* allele). The χ^2 value was 14.5 ($p<0.01$).

Back cross hybrid genotype	Male *C. gariepinus* (F)	Female F_1 big-oui hybrid (F/S)
Expected	F/F:	F/S
	14.5:	14.5
Observed	F/F:	F/S
	0:	29

Some morphological characters of live specimens were examined during this study, the results are shown in table 4.4 and figure 4.9. Both hybrids have intermediate morphology between the parental species, particularly for the occipital process and body coloration. A few reciprocal hybrids were reared until two months, when some morphological characters were examined. The results are shown in table 4.4.

4.3.6 Effect of using different stocks of male *C.gariepinus* on fertilisation and survival of hybrid

The fertilisation rates, survival rates to somite stage, hatch out and 3 days of age in big-oui hybrid using three difference original stocks of male *C. gariepinus* from 4 replicate experiments are shown in Table 4.5. The big-oui hybrid using the male imported stocks from Thailand had higher fertilisation and survival rates at the various development stages than the hybrids produced by using the two other stocks of male fish. Only the survival rate to the somite stage of development of all hybrid crosses did not show a significant difference. The fertilisation rate and survival in the hybrid produced by using the males from Wageningen stock was always significantly lower at every stage of larval development.

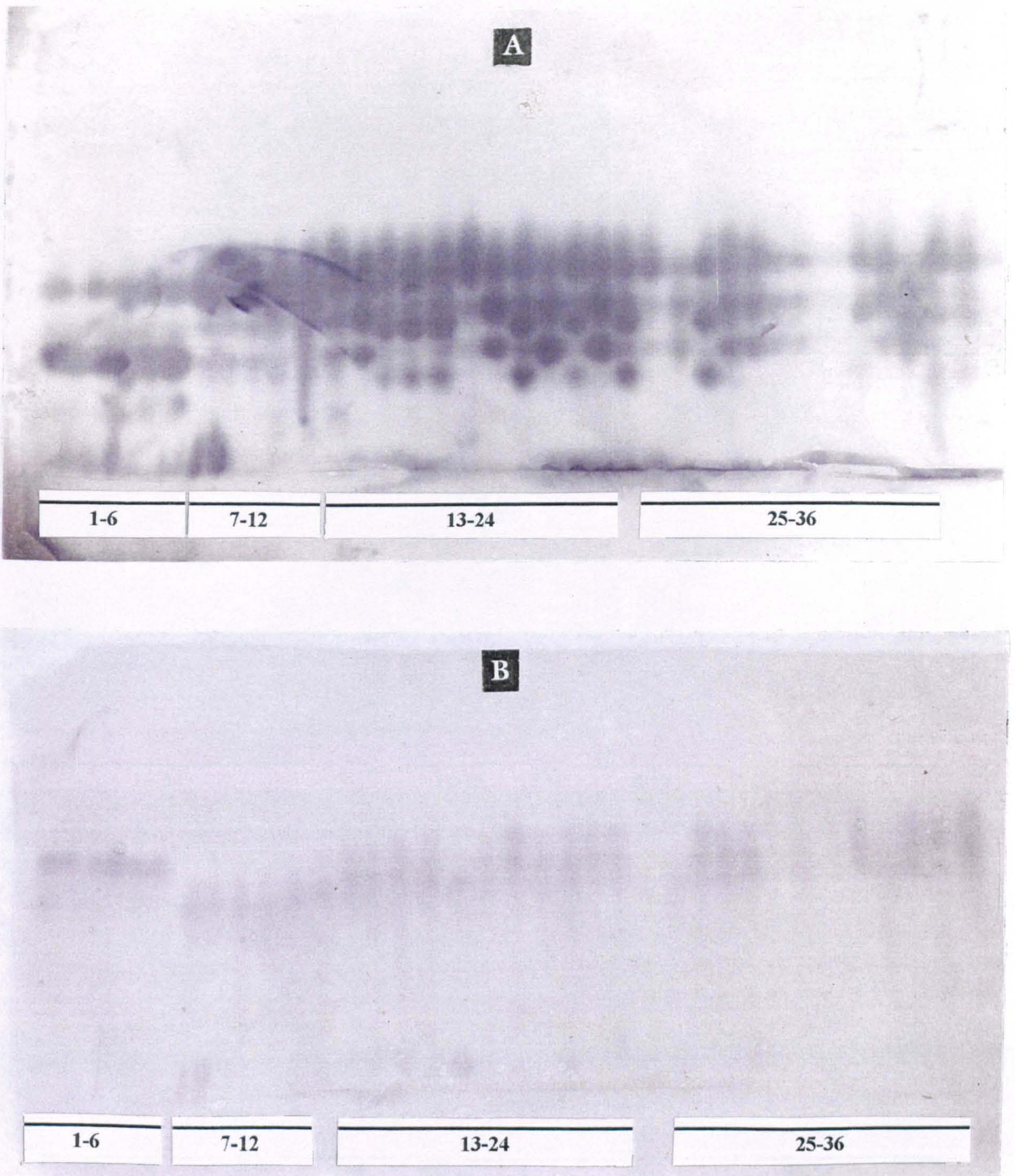


Figure 4.7 Diagnostic loci observed in larvae tissue of 12 big-oui hybrid (No.13-24) and 12 reciprocal cross hybrid (No.25-36) compared with muscle tissue from 6 *C. gariepinus* (No. 1-6) and 6 *C. macrocephalus* (No.7-12):- A) *GPI-2**,dimer and B) *MDH-2**, dimer.

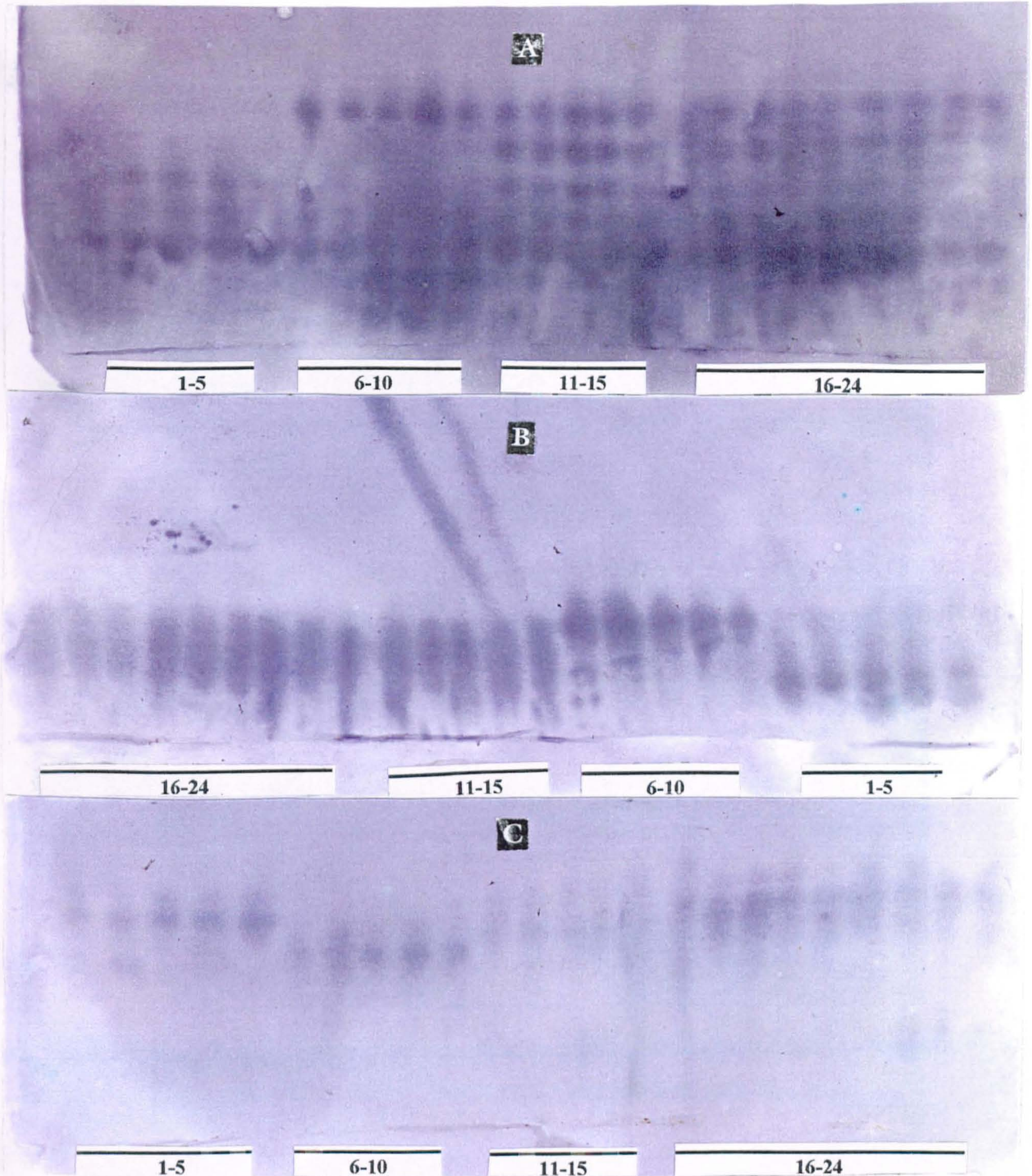


Figure 4.8 Diagnostic loci observed in muscle tissue of 5 *C. garirpinus* (No.1-5), 5 *C. macrocephalus* (No.6-10), 5 big-oui hybrid (No.11-15) and 9 back cross hybrid (No.16-24): A) *GPI-2** dimer, B) *LDH-1** tetramer and C) *MDH-2** dimer.

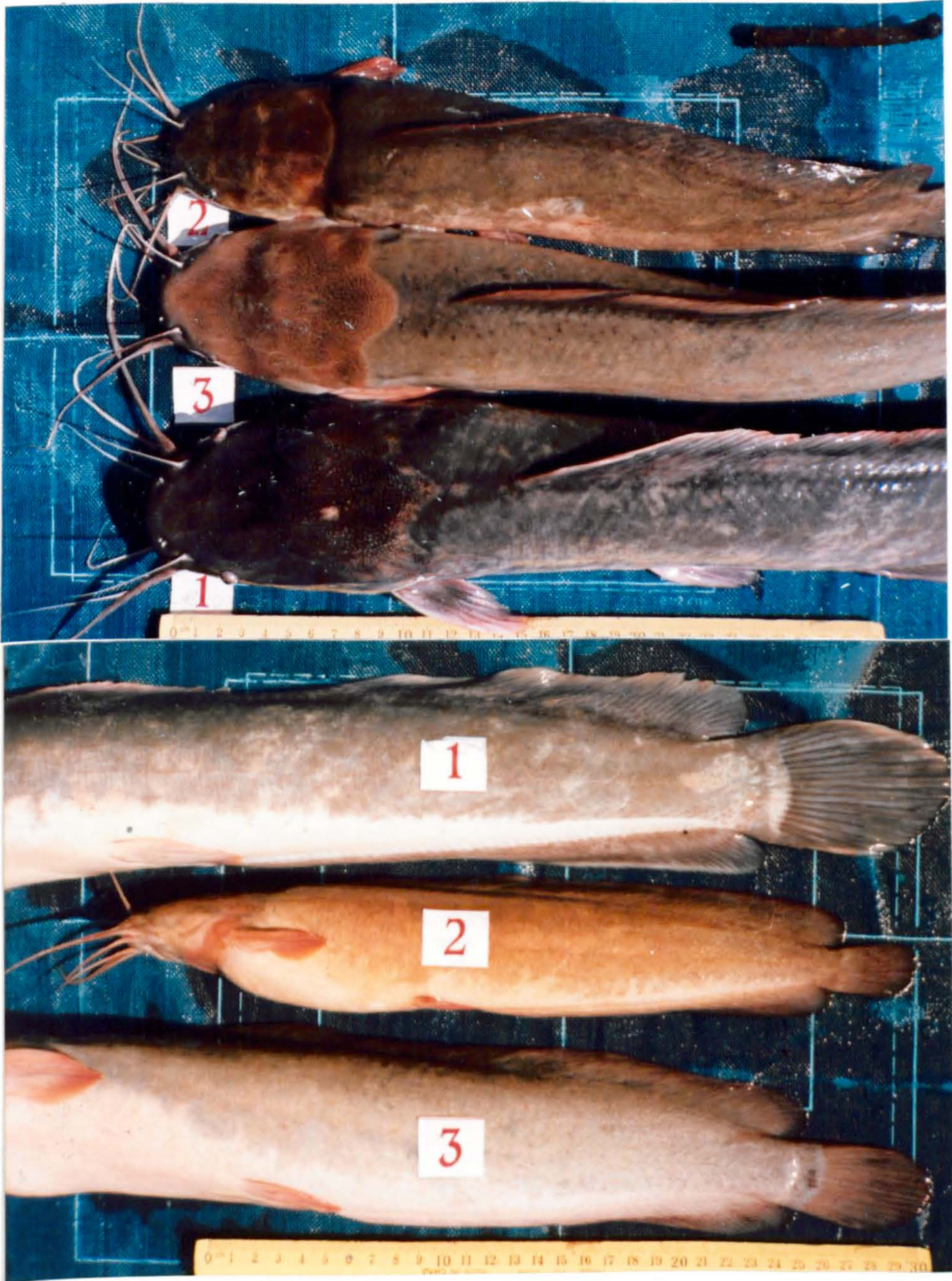


Figure 4.9 Shows pure *C. gariepinus* (1), Pure *C.macrocephalus* (2) and hybrid (3) showing most obvious morphological differences (a) shows the differences in the occipital process among pure species and hybrid (b) shows the differences in body coloration.

Table 4.4. Some morphological characters of Clariid catfishes studied in this chapter.

Characters	*C. macrocephalus*	*C. gariepinus*	Big-oui hybrid *C. macrocephalus (F)* × *C. gariepinus (M)*	Reciprocal hybrid cross *C. macrocephalus (M)* × *C. gariepinus (F)*
1. Shape of Body	Rather short (compared with *C. gariepinus*); body depth 5-6 times in total length	More slender (compared with *C. macrocephalus*); body depth 6-7 times in total length	Rather short (compared with *C. gariepinus*), more slender than *C. macrocephalus* but shorter than *C. gariepinus*; body depth 5-7 time in total length	Rather short (compared with *C. gariepinus*), shorter than big-oui hybrid; body depth 5-6 time in total length
2. Head				
2.1 Frontal part of head	Rather convex	Distinctly flattened	Not much flattened	Not much flattened
2.2 Upper surface of head	Smooth	Distinctly granulated	Granulated, more distinctly in bigger fish	Granulated
3 Occipital Process	Wide, low, broadly curved, its based width 3-5 times its length, extending close to the dorsal fin	Sharply angular	Angular, intermediate between *C. macrocephalus* and *C. gariepinus*	Angular, intermediate between *C. macrocephalus* and *C. gariepinus*
4. Pectoral Spine	Not serrated on the outer border	Serrated on the outer border	Serrated on the outer border	Serrated on the outer border
5. Colour Pattern				
5.1 Body	Nearly dark, with tiny white spots in vertical lines	Olive above, marbled with dark brown, white below	Marbled with dark brown above, white below; vertical lines of tiny white spots in young fish	Marbled with dark brown above, white below; with tiny white spots in vertical lines in young fish
5.2 Caudal Fin	Without vertical bar on the base of caudal fin	With light vertical bar on the base of caudal fin	With light vertical bar on the base of caudal fin, quite distinct in young fish but fainter in older fish	With light vertical bar on the base of caudal fin, quite distinct in young fish but fainter in older fish
6. Distance from Dorsal Fin to Occipital Process	Contained 5-7 times the length of the head (measured along upper median line)	3.5-5 times	Less than 5 times	Less than 5 times
7. Fin Rays				
7.1 Dorsal	62-70 (variable)	68-79 (variable)	67-70 (variable)	67-70 (variable)
7.2 Anal	45-50 (variable)	49-60 (variable)	50-54 (variable)	50-55 (variable)

Table 4.5 Mean survival rate of hybrid offspring produced by using different stocks of male *C. gariepinus* (data calculated from 4 replicated experiments).

Pair crosses	%±SE			
male(stock) × female	Fertilisation rate	Somite stage	Hatch out	3 days of age
Cg(TH)×Cm	87.08±0.03[b]	57.09±0.13[a]	51.16±0.07[b]	43.08±0.01[c]
Cg(MA)×Cm	81.79±0.05[ab]	49.36±0.17[a]	45.36±0.17[ab]	30.55±0.09[b]
Cg(WA)×Cm	74.52±0.12[a]	45.17±0.34[a]	37.44±0.06[a]	19.62±0.11[a]
±SEM	±0.02	±0.07	±0.03	±0.02

Data in the same column carrying different superscripts denote significant difference by ANOVA between values in each development stage at 95 % confident level.

Abbreviations; Cg(TH): *C.gariepinus* original imported stock from Thailand.

Cg(MA): *C.gariepinus* original imported stock from Malawi.

Cg(WA): *C.gariepinus* original imported stock from Wageningen, the Netherlands.

Cm: *C.macrocephalus* imported stock from Thailand.

4.4 Discussion.

Hybrid crosses between Thai native catfish (*C. macrocephalus*) and the African sharptooth catfish (*C. gariepinus*) were accomplished and analysed in this study. The karyotype of the big-oui hybrid, reciprocal crosses hybrid and back crosses hybrid were prepared and revealed each to have 2n = 55 chromosomes. Chromosomes of the parental species, 2n = 54 in *C. macrocephalus* and 2n = 56 in *C. gariepinus*, could be distinguished in the hybrid. For all hybrid crosses, the starch gel electrophoresis of *GPI-2**, *LDH-1** and *MDH-2** confirmed true hybridization. These results correspond with morphometrical data. Most morphological characteristic counts were intermediate between parental counts. The occipital process features in hybrids were intermediate between those of the parental species. A surprising result was that the back cross hybrids were heterozygotes for the three diagnostic loci (as observed in F_1 big-oui hybrid), different from the expected 1:1 homozygotes:heterozygotes.

Hybrids, which are intermediate in size and quality of flesh revealed features of maternal species, are cultured on a commercial scale. The fish flesh quality of this hybrid is better than that of *C. gariepinus*. A similar result was reported by Teugels *et al.* (1992b), where the hybrids between *C. gariepinus* and *Heterobranchus longifilis* were shown to be intermediate between the parental species. The hybrids of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) have increased yield, dressing percentage and catchability over those of the parental species (Yant *et al.*,1975; Chappell, 1979; and Tave *et al.*, 1981 cited by Krasznai, 1987). The hybrid of channel x blue catfish tolerated seawater longer than the parental species (Stickney and Simco, 1971). Dunham and Smitherman (1981) investigated growth in response to winter feeding and found that the outstanding winter growth of white catfish (*I. catus*) was not transmitted to channel x white catfish or white x blue hybrids. Winter growth

exhibited by all hybrids was less than of white catfish and the same as that of the channel and blue catfish. Na-Nakorn, *et al.* (1993a) found two morphotypes (confirmed by karyotyping) in first generation offspring from the artificial hybridization between *Clarias macrocephalus* and *Pangasius sutchi*. Understanding the big-oui hybrid characteristics should, in addition to determining the potential for aquaculture, contribute to current efforts to clarify the role of the parental species.

The hybrid of carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) showed more carp-like features. This hybrid has been reported to be fertile and to be able to back-cross (Hume *et al.*, 1983). The F_1 generations of silver carp and bighead carp reciprocal hybrids showed a heterosis effect with strong maternal dominance in their morphology (Bartholomew and Smitherman, 1984). The catla x rohu hybrid (*Catla catla* x *Labeo rohita*) showed intermediary inheritance in most features, the growth rate of the hybrid was greater than that of the rohu (Bhowmick *et al.*, 1981).

Since big-oui hybrid has been produced, only a few topics have been studied, and these are limited to some aspects of biology, such as morphology, growth rate and culture qualities. With the availability of a number of ponds at the National Inland Fisheries Institute, Thailand, research has been initiated for screening the qualities of promising hybrids for aquaculture. Culture of hybrid with parent species have been initiated for assessing the performance of this hybrid (Nukwan *et al.*, 1990). The success achieved in gynogenesis and androgenesis of several fishes have paved way for rapid genetic improvement of hybrids by intergeneric hybridization among the highly inbred, pure parental lines of culturable species. This is expected to yield valuable hybrid lines exhibiting a very high growth rate and other qualities for culture superior to those of the parental species. Crossing of tetraploid female carp and diploid male cyprinids results in viable allotriploids,

while their reciprocal crossing produces non-viable, weak aneuploid hybrids (Bakos *et al.*, 1978; Marian *et al.*, 1985). Carp x silver carp triploid hybrids were sterile fish with high productivity and good flesh quality, showing strong maternal (carp) dominance (Bakos *et al.*,1978).

One reason for producing artificial hybrids has been to obtain sterile fishes for aquaculture. However various papers have described maturation of hybrids and subsequent crosses, usually back crosses to one or both of the parental species (Naevdal and Dalpadado, 1987). The results from this study showed that both of big-oui hybrids and back cross hybrids were fertile, especially female big-oui hybrids which were able to back-cross to *C. gariepinus*. Lincoln (1981a,b) found that males predominated among plaice x flounder hybrids and six out of nine diploid hybrids yielded milt with mobile spermatozoa, all female hybrids were mature normally. Naevdal and Dalpadado (1987) suggested that crosses with a high degree of survival also generally had a good chance of producing fertile gametes. For example, both male and female gametes of crosses between *Oncorhynchus masou* and *O. rhodurus* were about equally viable as those of the parent species.

At this moment, the conclusion must be that in spite of clear advantages of the big-oui hybrids in intensive aquaculture, lack of interest in the pure native species (especially *C. batrachus*) may have deteriorated effects on conservation. This conclusion is supported by the fact that big-oui hybrids appear to be fertile, and they are able to back cross to the paternal species. For stocking purpose, the big-oui hybrid should not be stocked in natural water, if in the future they can adopt their natural breeding habit of *C. gariepinus*. However, triploid hybrids are observed to be sterile and more reliable than their diploid counterparts in this case (Purdom, 1972; Lincoln, 1981a; Chevassus, 1983). Genetic manipulations investigated on promising hybrids are being carried out simultaneously.

Production of sterile hybrids by intergeneric hybrization will be intensified and attempted for other species in Thailand as well.

The production of big-oui hybrids by using the male fish from Malawi stock and Wageningen stock gave lower fertilisation and survival than big-oui hybrid produced by using the male fish from Thailand. One reason could be suggested to explain this: the males from both stocks were very old (more than 8 years).

The F_2 hybrid never gave development to hatch out stage and no viable embryos at all. This might be caused by a reduction in size and poor development of the male testes in F_1 hybrids (even when they were more than one year old) or caused by incompatible chromosome of $2n = 55$ in F_1 big-oui hybrid.

The low survival of the reciprocal hybrids and back cross hybrids may be due to the genetic incompatibility of the two parental species. The small number of hatchlings were deformed. The chromosomes in the parent species show differences in numbers and morphology (Fig. 4.2-4.3). This investigation showed that both reciprocal hybrids and the back cross hybrid have $2n = 55$, the same as the chromosome number of the big-oui hybrid, although the samples were collected from newly hatched and deformed embryos.

The reciprocal cross and back cross hybrids do not appear promising for aquaculture. These hybrids did not survive long enough to enable studies on their performing growth rate and their fertility. Only a small number of the back cross hybrid between female F_1 hybrid and male *C. gariepinus* produced in this study were viable for growing up to one year of age, but they did not reach maturity. The back cross hybrid phenotype from electrophoresis showed a difference from the expected 1:1 (all were heterozygotes). This might be a hybridogenetic effect. Hybridogenetic species in nature are invariably diploid and, indeed, triploidy

would seem to preclude hybridogenesis (Moore, 1984). In hybridogenetics, a reduction division occurs and haploid gametes are produced, but the same genome is always segregated, intact, into functional gametes. Thus, a single genome is inherited clonally (Moore, 1984; Quattro *et al.*, 1991). *Poecilliopsis monacha-lucida* is the classic example and serves to illustrate this mode of inheritance. This fish arose via hybridization between two bisexual species, *P. monacha* and *P. lucida*. The *monacha* genome is transmitted through the germ line, whereas the *lucida* genome is discarded each generation. *Poecilliopsis monacha-lucida* has been synthesized in the laboratory by crossing female *P. monacha* with male *P. lucida* (Schultz, 1973 cited by Moore, 1984). This cross results in only female offspring, which then reproduce by hybridogenesis. In nature *P. monacha-lucida* mates with males of *P. lucida*; thus, the genotype of an F_1 hybrid between *monacha* and *lucida* is perpetuated *ad infinitum* where a newly assorted *lucida* genome is combined with the clonally inherited *monocaha* genome in each generation (Moore, 1984). In this study, the back cross hybrids which were sexed (9 individuals) were of both sexes where four out of six males yielded testes with undeveloping spermatozoa and the another two had reduced testis size. All females had ovaries which were reduced in size and seem to be retarded in development. The another preliminary study needs to be extended in the future. The production of the back cross hybrid would be a convenient method of obtaining sterile progeny of the F_1 hybrid and need the futher investigation of induced triploidy in the back crosses.

C. macrocephalus does not breed in cultured ponds: its breeding conditions are limited and naturally the breeding period is confined to 3-4 months a year. There is no indication that the big-oui hybrid is suitable to be naturally bred. Hybridization between *C. macrocephalus* and *C. gariepinus* was initially attempted to see if hybrids could breed over a prolonged period and adopt the pond breeding habit of *C. gariepinus*. It was feared that such a situation would

lead to a deleterious effect on native fish populations. Research into the genetics of this group, including aspects such as chromosome manipulation, may help to solve such problems and conserve the genetic integrity of native species.

The big-oui hybrid is now the single biggest fresh-water aquaculture product in Thailand, combining the superior taste of the native *C. macrocephalus* with the faster growth rate and better survial in intensive ponds of *C. gariepinus*. Due to a huge demand for the native species, *C. macrocephalus* which is difficult to produce in a large number for food or for broodstock to produce hybrids there is now a shortage of this species. They are often reared on a fairly extensive basis in ricefields and the harvested fish of both sexes are sold as broodstock. The males cannot be used in hybrid production as the reciprocal cross does not survive. The implications of the results from this study suggested that more genetic research in the native stocks of *C. macrocephalus* should be investigated for solving the shortage of this species. Genetic knowledge including chromosome manipulation was required to solve this problem.

CHAPTER FIVE

TRIPLOIDY STUDIES IN THE BIG-OUI HYBRID

(FEMALE *CLARIAS MACROCEPHALUS* X MALE *C. GARIEPINUS*)

5.1 Introduction

Although polyploidy can be induced readily in a wide variety of plants by chemical and physical agents which affect spindle function, similar studies in animals are rare and restricted to only arthopods, amphibians and fishes. Polyploid fish are frequently viable, however, as evidenced by examples of spontaneous polyploidy (Allen and Stanley, 1978). There is considerable interest in the application of induced triploidy in fish culture. Temperature or pressure shocking of fertilized eggs to induce triploidy has been successful for many fish species (Table 1). Both methods (temperature and pressure shocking) have been used to induce triploidy in fish by inhibiting the extrusion of the second polar body during the second meiotic division of newly fertilized eggs (Thorgaard, 1983; Purdom, 1983). Triploidy has been successfully induced by immersion of fertilized eggs in a cold water bath shortly after fertilisation (Purdom, 1972; Valenti, 1975; Wolters *et al.*, 1981; Cassani and Caton, 1985). Induction of polyploidy has also been reported when fish eggs were treated shortly after fertilisation with cytochalasin B (Refstie *et al.*, 1977; Allen and Stanley, 1979) and colchicine (Smith and Lemoine, 1979).

Triploid individuals might be expected to be larger than their diploid counterparts, in accordance with the general hypothesis that nuclear size increases in proportion to chromosome number while the nuclear-cytoplasmic volume ratio is maintained. However, Swarup (1959) reported triploid threespine sticklebacks to be no larger than diploids. Even though the cellular size increased, the number of cells per organ decreased. Purdom (1976) and Valenti (1975) found induced triploid plaice-flounder hybrids and blue tilapia to be significantly larger than normal diploids. These triploids differed from triploid threespine sticklebacks in their indeterminate growth pattern.

Triploid fish are often sterile because gonadal development is blocked or retarded. The effect of induced triploidy is often different for the two sexes. Female triploids usually have very reduced ovaries with few, if any, vitellogenic eggs, and do not usually show any signs of maturation. Male fish often produce normal sized testes but these contain watery milt with low numbers of aneuploid sperm. Secondary sexual characteristics are often present because the testes appear to be endocrinologically competent. Therefore, culture of all female triploid fish might be more profitable than that of non-sterile diploid fish.

In salmonids, triploids have often been shown to survive better during early development than diploid hybrids, for example "tiger trout", a vigorous hybrid between female brown trout (*Salmon trutta*) and male brook trout (*Salvelinus fontinalis*) which has good growth rates but poor survival. Attempts to produce this hybrid have been plagued by low survival rates, averaging only five percent to the initiation of feeding. Triploidization using heat shock has increased the survival rate to an average of 34 %.

Both triploid and diploid tiger trout are sterile. These sterile hybrids, particularly the female, may devote the energy that would otherwise go into the production of large volumes of eggs into increased growth. The production of all-female populations of triploid tiger trout might be ideal in this regard (Scheerer and Thorgaard, 1989).

This study was undertaken in *Clarias* hybrid catfish to determine the optimum time after fertilisation of zygotes, the optimum temperature level and duration of thermal shock, which ensures 100 % triploidy rate and maximum survival. Also it compares growth and sex ratio of the triploid and diploid fish. In addition the triploidization techniques could be applied to later production of gynogenetic diploids.

5.2 Materials and methods

5.2.1 Origin of broodstock

Parental catfishes, male *C. gariepinus* and female *C. macrocephalus* used in these experiments were supplied from the hatchery of the National Inland Fisheries Institute, Thailand and reared separately in fibre glass tanks for about 3-5 months under ambient temperature (27±1°C) for the production of good quality eggs.

5.2.2 Induced spawning and fertilisation

Spawning was induced by injection of female *C. macrocephalus* with LHRH analog hormone and a dopamine antagonist (Nukwan *et al.*, 1990). After a latency time of 15-

18 hrs the eggs can be hand-stripped and fertilized with milt from macerated testis of male *C. gariepinus*. About 500-800 fertilized eggs per treatment were incubated at 27± 1°C in 20 liter plastic aquaria in a recirculating incubation system. The plastic aquaria contained small screens (0.5 mm^2 mesh-size) to support the developing eggs; hatched embryos can pass through this mesh allowing easy removal of unhatched eggs and egg debris.

5.2.3 Temperature shock (cold and heat)

Cold-shock and heat-shock experiments were carried out by transferring the screens with eggs into a controlled temperature water bath. For optimizing the cold shock, the temperature was varied from 1-8°C. These shocks were applied at 1-8 minutes after fertilisation at 1 minute intervals. The durations of the shocks were varied from 10- 30 minutes at 5 minutes intervals (Vejaratpimol and Pewnim, 1990; Richter *et al.*, 1987). For optimizing heat shock, the temperature was varied from 37-41°C, the times after fertilisation were varied from 2-8 minutes at 1 minute intervals and the durations of the shocks were varied from 2-5 minutes at 1 minutes intervals (the details of application were described in section 2.7.1).

5.2.4 Pressure shock

The hydrostatic pressure shock experiments were carried out by transferring the screens with fertilized eggs into a pressure vessel which had first been filled with clean water. After the vessels had been sealed and purged of air, the pressure release valve was

closed and pressure was applied gradually by a manually operated hydraulic pump. The pressure was varied from 8,000-10,000 p.s.i. at 1,000 p.s.i. intervals, the times after fertilisation were varied from 20-30 minutes at 2 minute intervals and the duration of the shocks were varied from 2-6 minutes at 1 minute intervals (the details of application as described in section 2.7.2).

5.2.5 Egg incubation and fry rearing

After the shocking, the eggs were transferred again to the recirculating water incubation system at 27±1°C. At about 22-26 hrs after fertilisation, hatching takes place. The larvae were subsequently reared in a recirculating water system at 27±1°C. They were fed with *Artemia* nauplii during the first two weeks after yolk sac resorption. After this the fry were fed with various sizes of trout pellet (commercial catfish pellets are not available in U.K).

5.2.6 Estimation of induction efficiency

The survival rate of the controls was defined as the percentage of eggs that developed into normal larvae. The survival rate of the treated eggs were expressed as a percentage of the survival rate of the controls. Triploidy rates were expressed as a percentage of the number of normal individuals examined.

5.2.7 Determination of ploidy

Cytogenetic method. 20 embryos from each treatment group were sampled, karyotypes were prepared from newly hatched or 1 day old larvae from each batch by colchicine treatment. Ploidy was determined by counting chromosomes per spread in larval tissue prepared according to the solid tissue technique of Kligerman and Bloom (1977). Metaphase chromosome spreads were checked and the frequency of individual chromosome counts were recorded, then the percentage of triploidy induction was calculated.

Erythrocyte nuclear volume method, one group each of triploid and control larvae from the optimized cold shock treatment was reared separately in 100 *l* aquaria until 6 months of age at a stocking rate of 25 fishes per aquarium for comparision of the growth rate. At the end of the experiment all fishes were killed for examination of the gonads and blood samples were drawn from the dorsal artery of the caudal fin. Blood smears were prepared and stained with Wright's stain. Major and minor axes of the nuclei of 10 erythrocytes from each individual fish were measured. Nuclear volume were calculated as 4/3 x ab^3, where a is the major, and b is the minor semi-axis (Wolters *et al.*, 1982b).

5.2.8 Comparison of the growth rate and feed ultilization between triploid and diploid hybrids.

The larvae from untreated and the optimized cold shock experiment were reared separately in two replicate 100 l glass aquaria until 6 months of age, at a stocking density of 25 fish per aquarium and were fed with commercial catfish pellets (available in Thailand; 40 % protein). The fish were fed *ad libitum* three times daily and may therefore be considered to be growing at the maximum rate. The mean fresh body weight and lenght were determined every two weeks. Finally, all fishes were used to determine the gonadosomatic index (GSI) and blood samples were drawn for erythrocyte nuclear volume measurement (see section 5.2.7) The data on survival rates, growth rate and GSI were analyzed with analysis of variance (ANOVA).

5.3.Results

5.3.1 Triploidy induction using temperature and pressure shocks

The results of the present study for the identification of treatments in inducing triploidy by exposing fertilized eggs of the big-oui hybrid to altered intensities, durations of shock and times after insemination of cold shock, heat shock and pressure shock are described. It is convenient to begin by first explaining the preliminary results of those experiments conducted in Thailand (1992-1993).

5.3.1.1 Cold shocks

The results of experiments performed using cold shock treatments are detailed in Table 5.1. The percentage of triploid yield compared with control diploid hybrids at different times after fertilisation and durations of cold shock are presented in Figure 5.1-5.3. Triploids were produced by all temperatures of cold shock in the range 1-8°C, at timings in the range of 1-8 mins after insemination and the shock duration of 10, 15, 20, 25 and 30 min. Any duration above this level resulted in lower survival rates which consequently lowered triploid rates. No triploids were produced when eggs were submitted to cold shocks of above 8°C for 30 mins duration. Earlier (1-5 mins TAF) and later (6-8 min TAF) application of every temperature and duration.

resulted in lower survival rates and triploid rates. Triploidy rates declined sharply (close to 0 %) with later (7-8 mins TAF) application of cold shock. At 2-5°C the most effective time of administration of 10, 15 and 20 min duration shock was 4 mins TAF to induce maximum triploid yield. Triploidy rate (100%) and survival rate to hatch (96.15%) were apparently highest yield after the application of 10 min duration at 4°C shock administered at 2 min TAF. Cold shock at 2°C for 15 min duration and 4 min TAF gave also a high yield with prolonged time of administration. (Table 5.1 and Figure 5.1-5.3). Although triploids were induced by the application of lower temperature (2-5°C), lower triploid yields were associated with increased numbers of deformed embryos at these temperatures. Higher temperatures of cold shock (6-8°C) were generally less effective in inducing triploidy.

Table 5.1 The effects of difference intensities, durations and timings of application of cold shock on survival rate and triploid rate in big-oui hybrid catfish.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate %	Survival rate % RC*	Triploid rate %	Triploid yield %
1	1	0	83.08	100	0	0
1	1	10	74.68	98.88	0	0
1	1	15	46.15	55.55	0	0
1	1	20	43.59	52.46	0	0
1	1	25	35.63	42.88	0	0
1	1	30	39.47	47.51	0	0
1	2	0	84.75	100	0	0
1	2	10	67.03	79.09	0	0
1	2	15	65.64	77.45	0	0
1	2	20	43.21	50.98	0	0
1	2	25	4.17	4.92	0	0
1	2	30	22.94	27.26	0	0
1	3	0	87.50	100	0	0
1	3	10	83.91	95.89	0	0
1	3	15	80.82	92.36	0	0
1	3	20	62.82	71.79	0	0
1	3	25	0	0	0	0
1	3	30	0	0	0	0
1	4	0	87.10	100	0	0
1	4	10	48.00	55.11	0	0
1	4	15	9.93	11.40	0	0
1	4	20	10.95	12.57	0	0
1	4	25	11.69	13.42	0	0
1	4	30	0	0	0	0
1	5	0	76.19	100	0	0
1	5	10	31.03	40.73	0	0
1	5	15	58.44	76.70	0	0
1	5	20	30.77	40.39	0	0
1	5	25	71.43	93.75	0	0
1	5	30	24.78	32.52	0	0
1	6	0	71.74	100	0	0
1	6	10	65.00	90.60	0	0
1	6	15	60.81	84.76	0	0
1	6	20	42.19	58.81	0	0
1	6	25	65.06	90.69	0	0
1	6	30	24.73	34.47	0	0
1	7	0	83.10	100	0	0
1	7	10	56.34	67.80	0	0
1	7	15	46.39	55.82	0	0
1	7	20	52.33	62.97	0	0
1	7	25	48.94	58.89	0	0
1	7	30	40.00	48.23	0	0
1	8	0	56.07	100	0	0
1	8	10	42.86	76.44	0	0
1	8	15	49.33	87.98	0	0
1	8	20	39.39	70.25	0	0
1	8	25	8.41	15.00	0	0
1	8	30	31.37	55.95	0	0

Table 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate %	Survival rate % RC*	Triploid rate %	Triploid yield %
2	1	0	72.13	100	0	0
2	1	10	60.19	83.45	0	0
2	1	15	55.14	76.45	0	0
2	1	20	0	0	0	0
2	1	25	0	0	0	0
2	1	30	0	0	0	0
2	2	0	87.50	100	0	0
2	2	10	82.69	94.50	0	0
2	2	15	78.57	89.79	0	0
2	2	20	67.52	77.17	0	0
2	2	25	47.54	54.33	0	0
2	2	30	31.58	36.09	0	0
2	3	0	71.43	100	0	0
2	3	10	41.09	57.52	0	0
2	3	15	30.10	34.32	50	17.16
2	3	20	35.44	40.40	40	16.16
2	3	25	35.95	40.98	20	8.200
2	3	30	5.93	6.76	0	0
2	4	0	76.72	100	0	0
2	4	10	75.68	98.64	70	69.05
2	4	15	73.37	95.63	100	95.63
2	4	20	44.69	58.25	100	58.25
2	4	25	60.25	78.53	100	78.53
2	4	30	75.93	98.98	80	79.18
2	5	0	73.72	100	0	0
2	5	10	49.15	66.67	50	33.34
2	5	15	17.99	24.40	60	14.64
2	5	20	33.83	45.89	80	36.71
2	5	25	14.09	19.11	100	19.11
2	5	30	0	0	0	0
2	6	0	78.44	100	0	0
2	6	10	66.14	84.32	20	16.86
2	6	15	64.82	82.63	20	16.53
2	6	20	70.15	89.43	80	71.54
2	6	25	35.17	44.84	60	26.90
2	6	30	25.31	32.27	100	32.27
2	7	0	74.88	100	0	0
2	7	10	46.75	62.44	0	0
2	7	15	39.00	52.08	40	20.83
2	7	20	12.32	16.45	60	9.87
2	7	25	9.29	12.41	100	12.41
2	7	30	0	0	0	0
2	8	0	70.75	100	0	0
2	8	10	22.64	32.00	0	0
2	8	15	28.57	40.38	0	0
2	8	20	29.52	41.72	0	0
2	8	25	25.23	35.66	0	0
2	8	30	20.37	28.79	0	0

Table 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate	Triploid yield
			%	% RC*	%	%
3	1	0	71.96	100	0	0
3	1	10	44.79	62.43	0	0
3	1	15	27.49	38.20	0	0
3	1	20	19.01	26.42	0	0
3	1	25	17.65	24.53	0	0
3	1	30	1.10	1.53	0	0
3	2	0	73.98	100	0	0
3	2	10	40.82	55.18	0	0
3	2	15	33.70	45.55	0	0
3	2	20	42.02	56.80	0	0
3	2	25	43.95	59.40	0	0
3	2	30	47.62	64.37	0	0
3	3	0	85.41	100	0	0
3	3	10	72.09	84.40	-	-
3	3	15	76.67	89.77	70	62.84
3	3	20	45.45	53.21	100	53.21
3	3	25	41.38	48.45	100	48.45
3	3	30	28.00	32.78	100	32.78
3	4	0	82.86	100	0	0
3	4	10	82.15	99.14	0	0
3	4	15	60.12	72.55	40	29.02
3	4	20	54.67	65.98	100	65.98
3	4	25	61.29	73.96	50	36.98
3	4	30	45.06	54.38	100	54.38
3	5	0	88.64	100	0	0
3	5	10	72.73	82.05	0	0
3	5	15	57.14	64.46	10	6.45
3	5	20	65.00	73.73	80	58.66
3	5	25	40.00	45.12	0	0
3	5	30	0	0	0	0
3	6	0	82.01	100	0	0
3	6	10	74.62	90.99	0	0
3	6	15	73.16	89.21	0	0
3	6	20	66.93	81.61	0	0
3	6	25	47.67	58.13	0	0
3	6	30	54.99	67.05	0	0
3	7	0	70.42	100	0	0
3	7	10	38.18	54.22	0	0
3	7	15	18.69	26.54	0	0
3	7	20	2.99	4.25	0	0
3	7	25	0	0	0	0
3	7	30	0	0	0	0
3	8	0	84.29	100	0	0
3	8	10	79.75	94.62	0	0
3	8	15	40.37	47.89	0	0
3	8	20	25.68	30.46	0	0
3	8	25	14.29	16.95	0	0
3	8	30	4.23	5.02	0	0

Table 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate	Triploid yield
			%	% RC*	%	%
4	1	0	85.66	100	0	0
4	1	10	80.43	93.89	0	0
4	1	15	43.08	50.29	0	0
4	1	20	34.76	40.58	0	0
4	1	25	22.22	25.94	0	0
4	1	30	10.93	12.76	0	0
4	2	0	94.55	100	0	0
4	2	10	90.91	96.15	100	96.15
4	2	15	88.24	93.32	100	93.32
4	2	20	51.78	54.76	100	54.76
4	2	25	12.56	13.28	40	5.31
4	2	30	1.48	1.58	0	0
4	3	0	87.71	100	0	0
4	3	10	78.79	89.83	100	89.83
4	3	15	24.24	27.64	100	27.64
4	3	20	41.38	47.18	100	47.18
4	3	25	7.19	8.20	66.67	5.46
4	3	30	10.11	11.53	70	8.07
4	4	0	85.00	100	0	0
4	4	10	80.65	94.88	100	94.88
4	4	15	48.39	56.93	20	11.39
4	4	20	53.33	62.74	100	62.74
4	4	25	17.86	21.01	60	12.61
4	4	30	22.22	26.14	0	0
4	5	0	69.81	100	0	0
4	5	10	67.74	97.04	0	0
4	5	15	48.86	69.99	0	0
4	5	20	43.93	62.93	0	0
4	5	25	12.36	17.71	0	0
4	5	30	18.00	25.78	0	0
4	6	0	76.92	100	0	0
4	6	10	46.00	52.00	0	0
4	6	15	30.00	39.00	0	0
4	6	20	64.29	83.58	0	0
4	6	25	4.19	5.45	0	0
4	6	30	0	0	0	0
4	7	0	76.19	100	0	0
4	7	10	71.67	94.07	0	0
4	7	15	35.29	46.32	0	0
4	7	20	17.20	22.58	0	0
4	7	25	0	0	0	0
4	7	30	0	0	0	0
4	8	0	96.97	100	0	0
4	8	10	71.19	73.41	0	0
4	8	15	60.00	61.88	0	0
4	8	20	17.24	17.78	0	0
4	8	25	23.81	24.55	0	0
4	8	30	20.00	20.63	0	0

TOable 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate	Triploid yield
			%	% RC*	%	%
5	1	0	65.05	100	0	0
5	1	10	60.90	93.62	0	0
5	1	15	54.21	83.34	0	0
5	1	20	28.10	43.20	0	0
5	1	25	4.61	7.09	0	0
5	1	30	3.08	4.74	0	0
5	2	0	79.66	100	0	0
5	2	10	53.15	66.72	50	33.36
5	2	15	46.67	58.59	0	0
5	2	20	47.49	59.62	100	59.62
5	2	25	32.96	42.10	0	0
5	2	30	8.49	10.85	0	0
5	3	0	90.60	100	0	0
5	3	10	81.65	90.12	100	90.12
5	3	15	71.59	79.02	40	31.61
5	3	20	66.82	73.75	100	73.75
5	3	25	65.90	72.74	0	0
5	3	30	71.23	78.62	0	0
5	4	0	80.16	100	0	0
5	4	10	76.92	95.96	0	0
5	4	15	62.29	77.71	100	77.71
5	4	20	73.68	91.92	100	91.92
5	4	25	57.42	71.63	30	21.49
5	4	30	44.98	56.11	20	11.22
5	5	0	88.24	100	0	0
5	5	10	81.87	92.78	100	92.78
5	5	15	69.11	78.32	30	23.50
5	5	20	70.76	80.19	50	40.10
5	5	25	33.33	37.77	40	15.11
5	5	30	8.82	9.99	0	0
5	6	0	90.44	100	0	0
5	6	10	86.21	95.32	0	0
5	6	15	70.89	78.38	100	78.38
5	6	20	52.63	58.19	50	29.10
5	6	25	56.57	62.55	80	50.04
5	6	30	47.17	52.16	0	0
5	7	0	74.09	100	0	0
5	7	10	25.83	34.86	0	0
5	7	15	20.53	27.71	0	0
5	7	20	6.45	8.71	0	0
5	7	25	6.90	9.31	0	0
5	7	30	4.17	5.63	0	0
5	8	0	81.48	100	0	0
5	8	10	24.36	29.90	0	0
5	8	15	26.72	32.79	0	0
5	8	20	7.43	9.12	0	0
5	8	25	0	0	0	0
5	8	30	0	0	0	0

Table 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate %	Triploid yield %
			%	% RC*		
6	1	0	65.22	100	0	0
6	1	10	60.46	92.70		0
6	1	15	38.10	58.42	0	0
6	1	20	33.01	50.61	0	0
6	1	25	19.05	29.21	0	0
6	1	30	0	0	0	0
6	2	0	82.73	100	0	0
6	2	10	36.21	43.77	0	0
6	2	15	24.67	29.82	0	0
6	2	20	2.53	3.06	0	0
6	2	25	16.29	19.69	0	0
6	2	30	0	0	0	0
6	3	0	83.33	100	0	0
6	3	10	27.50	33.00	20	6.60
6	3	15	32.43	38.92	30	11.68
6	3	20	33.33	40.00	0	0
6	3	25	56.41	67.70	60	40.62
6	3	30	24.44	29.33	0	0
6	4	0	100	100	0	0
6	4	10	76.92	76.92	100	76.92
6	4	15	69.50	69.50	100	69.50
6	4	20	57.14	57.14	60	34.28
6	4	25	44.90	44.90	10	4.49
6	4	30	35.29	35.29	0	0
6	5	0	70.46	100	0	0
6	5	10	20.45	29.02	0	0
6	5	15	24.73	35.10	20	7.02
6	5	20	18.52	26.28	10	2.63
6	5	25	8.54	12.12	0	0
6	5	30	0	0	0	0
6	6	0	89.13	100	0	0
6	6	10	88.16	98.91	0	0
6	6	15	80.67	90.51	0	0
6	6	20	21.67	24.31	0	0
6	6	25	10.07	11.30	0	0
6	6	30	0	0	0	0
6	7	0	74.87	100	0	0
6	7	10	44.19	59.02	0	0
6	7	15	35.45	47.35	0	0
6	7	20	32.11	42.89	0	0
6	7	25	0	0	0	0
6	7	30	0	0	0	0
6	8	0	71.90	100	0	0
6	8	10	38.10	52.99	0	0
6	8	15	23.15	32.20	0	0
6	8	20	23.08	32.10	0	0
6	8	25	12.28	17.08	0	0
6	8	30	0	0	0	0

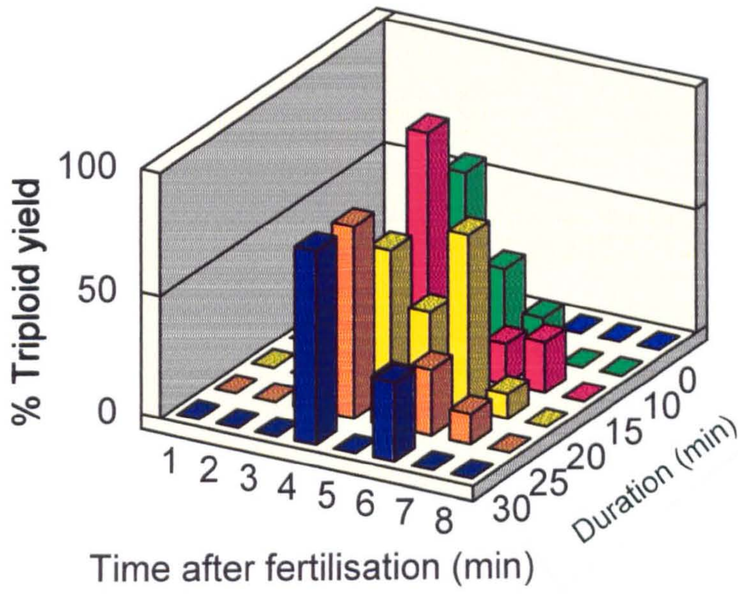
Table 5.1 cont.

Temperatur (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate %	Triploid yield %
			%	% RC*		
7	1	0	80.00	100	0	0
7	1	10	44.44	55.55	0	0
7	1	15	40.89	51.11	0	0
7	1	20	39.78	49.73	0	0
7	1	25	29.26	36.58	0	0
7	1	30	11.86	14.83	0	0
7	2	0	70.77	100	0	0
7	2	10	32.93	36.53	0	0
7	2	15	26.32	37.19	0	0
7	2	20	14.02	19.81	0	0
7	2	25	19.61	27.71	0	0
7	2	30	12.12	17.13	0	0
7	3	0	82.80	100	0	0
7	3	10	78.36	94.64	0	0
7	3	15	69.77	84.26	0	0
7	3	20	57.14	69.01	0	0
7	3	25	58.63	70.81	0	0
7	3	30	46.15	55.74	0	0
7	4	0	85.09	100	0	0
7	4	10	70.57	82.94	0	0
7	4	15	72.97	85.76	20	17.15
7	4	20	22.44	26.37	50	13.19
7	4	25	1.75	2.06	0	0
7	4	30	0	0	0	0
7	5	0	89.20	100	0	0
7	5	10	87.80	98.43	0	0
7	5	15	84.97	95.26	20	19.05
7	5	20	53.19	59.63	40	23.85
7	5	25	6.89	7.72	0	0
7	5	30	0	0	0	0
7	6	0	90.57	100	0	0
7	6	10	53.58	59.16	20	11.83
7	6	15	44.20	48.80	0	0
7	6	20	27.45	30.31	0	0
7	6	25	20.00	22.08	0	0
7	6	30	0	0	0	0
7	7	0	80.00	100	0	0
7	7	10	67.55	84.44	0	0
7	7	15	45.10	56.38	0	0
7	7	20	13.54	16.93	0	0
7	7	25	2.88	3.60	0	0
7	7	30	0	0	0	0
7	8	0	75.97	100	0	0
7	8	10	65.42	86.11	0	0
7	8	15	35.16	46.28	0	0
7	8	20	1.81	2.38	0	0
7	8	25	0	0	0	0
7	8	30	0	0	0	0

Table 5.1 cont.

Temperature (°C)	Time after fertilisation (min)	Duration (min)	Survival rate		Triploid rate %	Triploid yield %
			%	% RC*		
8	1	0	91.78	100	0	0
8	1	10	68.81	74.97	0	0
8	1	15	4.63	5.05	0	0
8	1	20	2.33	2.54	0	0
8	1	25	0	0	0	0
8	1	30	0	0	0	0
8	2	0	70.48	100	0	0
8	2	10	57.62	81.75	0	0
8	2	15	39.02	55.36	0	0
8	2	20	16.83	23.88	0	0
8	2	25	0	0	0	0
8	2	30	0	0	0	0
8	3	0	84.54	100	0	0
8	3	10	78.13	92.42	0	0
8	3	15	28.57	33.80	0	0
8	3	20	16.67	19.72	0	0
8	3	25	6.02	7.12	0	0
8	3	30	0	0	0	0
8	4	0	90.70	100	0	0
8	4	10	38.81	42.79	0	0
8	4	15	34.08	37.57	0	0
8	4	20	34.29	37.81	0	0
8	4	25	9.19	10.13	0	0
8	4	30	0	0	0	0
8	5	0	77.27	100	0	0
8	5	10	39.75	51.44	0	0
8	5	15	33.67	43.57	0	0
8	5	20	30.92	40.02	0	0
8	5	25	8.16	10.56	0	0
8	5	30	0	0	0	0
8	6	0	82.50	100	0	0
8	6	10	36.84	44.66	0	0
8	6	15	22.22	26.93	0	0
8	6	20	13.04	15.81	0	0
8	6	25	27.59	33.44	0	0
8	6	30	0	0	0	0
8	7	0	91.35	100	0	0
8	7	10	58.61	64.16	0	0
8	7	15	42.33	46.34	0	0
8	7	20	13.51	14.79	0	0
8	7	25	0	0	0	0
8	7	30	0	0	0	0
8	8	0	88.24	100	0	0
8	8	10	58.45	66.24	0	0
8	8	15	40.53	45.93	0	0
8	8	20	18.81	21.32	0	0
8	8	25	0	0	0	0
8	8	30	0	0	0	0

RC* Relative to control after adjustment of the latter to 100%.



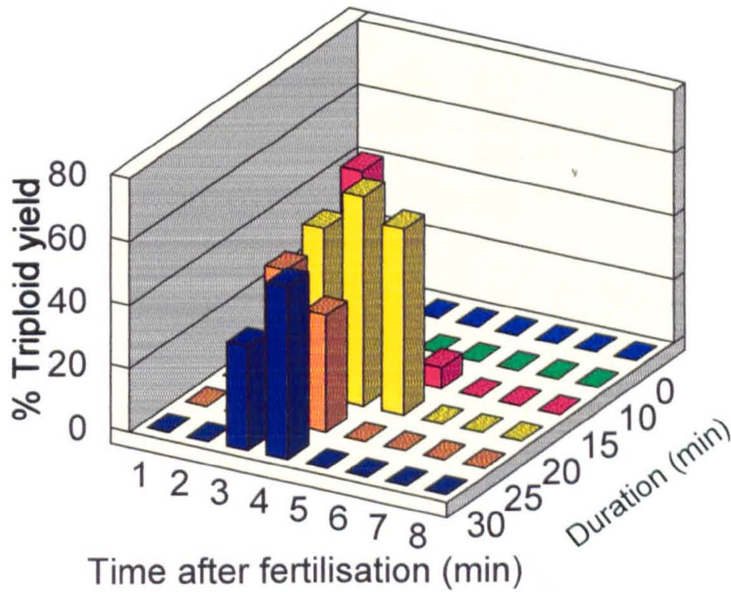
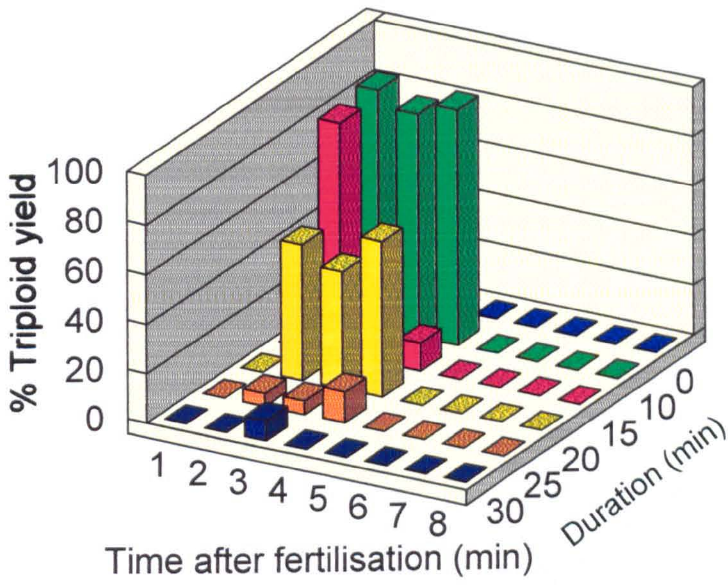


Figure 5.1 Triploid yield (%) derived from cold shock at varied times after fertilisation (1-8 min) and durations (10, 15, 20, 25, and 30 min) A) cold shock at 2°C B) cold shock at 3°C.



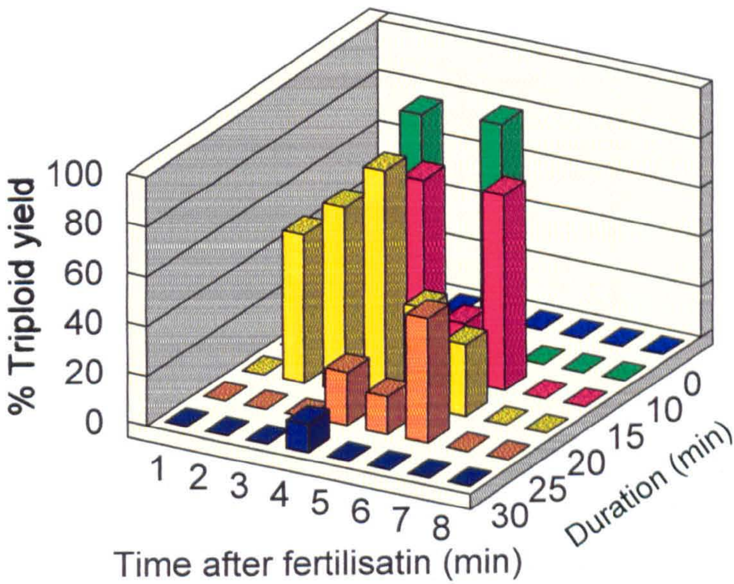


Figure 5.2 Triploid yield (%) derived from cold shock at varied times after fertilisation (1-8 min) and durations (10, 15, 20, 25, and 30 min) A) cold shock at 4°C B) cold shock at 5°C.

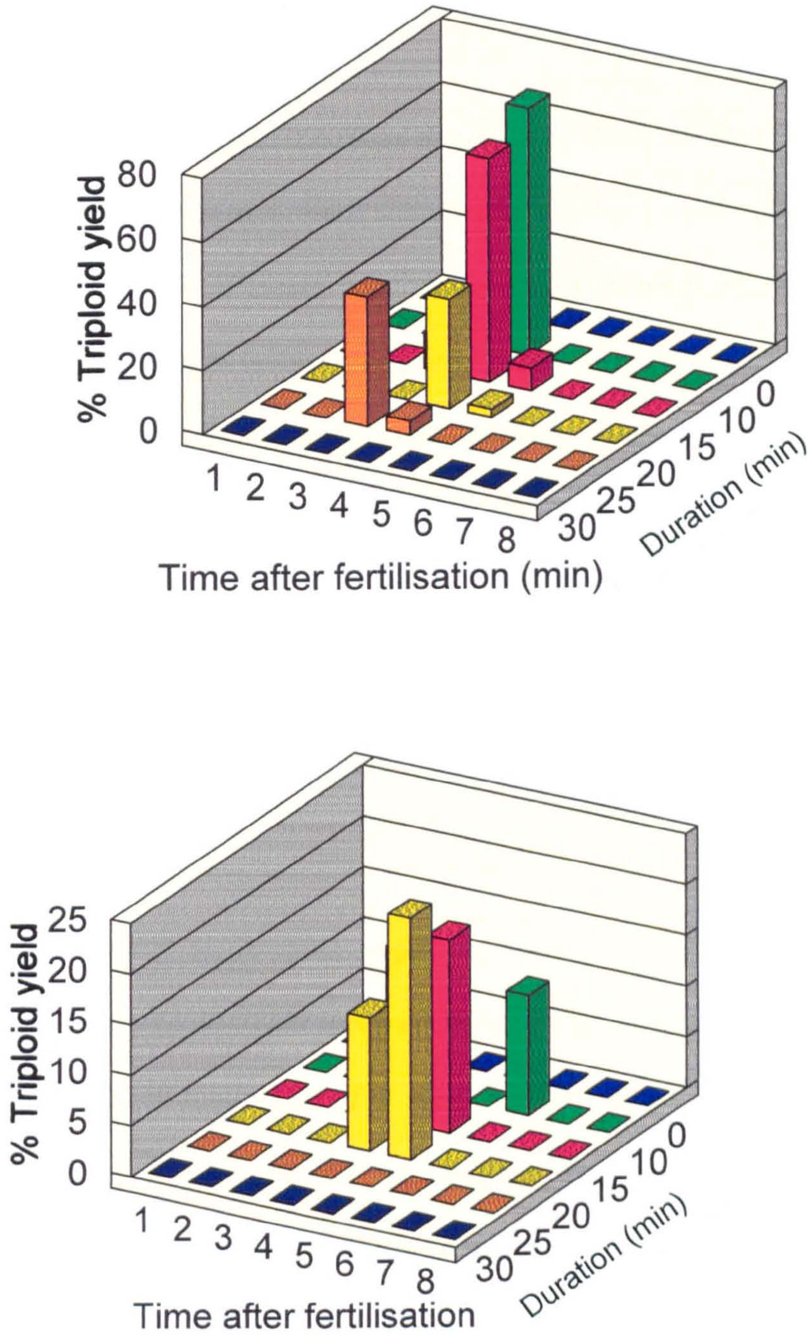


Figure 5.3 Triploid yield (%) derived from cold shock at varied times after fertilisation (1-8 min) and durations (10, 15, 20, 25, and 30 min) A) cold shock at 6° C B) cold shock at 7°C

5.3.1.2 Heat shock

The results of heat shock experiments are presented in Table 5.2. Heat shocks at 38, 39, 40 and 41°C were applied at 4, 5 and 6 mins TAF and for 5 or 10 mins duration. The heat shocks, even those of longer duration were more effective on survival rates but were not very effective in inducing triploidy. Increased temperatures and duration of heat shocks were effect to low survival rate of larvae. The most effective temperature of shock was 38°C, administered 5 mins TAF and 5 mins shocking duration. This dose gave the highest survival rate of embryos and a high triploid rate. Shocking temperature higher than 40°C were less effective because they produced lower survival rate.

5.3.1.3 Pressure shock

The results of the pressure shock experiments are presented in Table 5.3. These preliminary results, showed poor survival rates and triploid rates of following pressure level and timings of application of 2 mins duration shocks at 4-8 mins TAF and temperature of 28°C. All levels of 2 mins duration shock indicated very low survival rate to hatch of embryos, From this, it can be assumed that pressure shocks in this range are not suitable for inducing triploidy in *Clarias* catfish.

Table 5.2 The effects of different intensities, durations and timings of application of heat shock on survival rate and triploids rate in big-oui hybrid catfish.

T (^{o}C)	TAF (min)	D (min)	Survival rate (%±SD)	%RC* (%±SD)	Triploidy rate (%±SD)	Triploid yield (%±SD)
28 (control)	0	0	73.44±8.52	100±0.0	0	0
38	4	5	39.06±10.34	52.26±8.02	80±20	40.20±4.04
	4	10	26.11±5.67	35.13±3.65	85±5	30.04±4.86
	5	5	42.02±5.34	57.14±0.64	90±10	51.36±5.14
	5	10	18.58±2.15	25.30±0.01	100±0.0	25.30±0.01
	6	5	33.55±4.87	45.53±1.35	80±0.0	36.42±1.08
	6	10	20.12±2.52	27.37±0.26	80±10	21.87±2.54
39	4	5	16.42±7.34	21.49±7.50	80±10	17.94±8.15
	4	10	11.04±6.60	14.15±7.62	100±0.0	14.15±7.62
	5	5	15.66±4.55	20.89±3.78	75±5	15.66±1.97
	5	10	0.37±0.37	0.45±0.45	0	0
40	4	5	4.62±1.68	6.11±1.58	80#	6.15
	4	10	1.51±0.59	1.99±0.57	65±5	1.32±0.47
	5	5	2.32±0.38	3.14±0.15	85±5	2.68±0.29
	5	10	0.85±0.65	1.07±0.76	100#	1.83
41	4	5	2.64±1.68	3.38±1.90	80±0.0	2.70±1.52
	4	10	3.89±1.44	5.14±1.37	75±5	3.79±0.77
	5	5	1.95±1.95	2.38±2.38	40#	1.90
	5	10	0	0	0	0

Experiments were conducted using 2 different females as replications.

RC* Relative to control after adjustment of the latter to 100%.

Missing data in one replication.

Table 5.3 The effects of different intensities, durations and timings of application of pressure shock on survival to hatch and triploidy rates in big-oui hybrid catfish.

Pressure (p.s.i.)	TAF (min)	D (min)	Survival rate (%±SD)	Survival rate (%RC*±SD)	Triploidy rate (%±SD)	Triploid yield (%±SD)
control	0	0	57.4±12.5	100	0	0
7000	4	2	0	0	0	0
	5	2	0	0	0	0
	6	2	0.87±0.54	1.38±0.65	0	0
	7	2	4.17±0.36	7.49±1.01	25±5	1.96±0.63
	8	2	4.33±2.51	6.92±2.87	35±15	2.39±1.58
8000	4	2	0	0	0	0
	5	2	1.66±0.53	2.83±0.31	20±10	0.60±0.35
	6	2	2.89±1.37	4.74±1.35	35±15	1.87±1.19
	7	2	3.90±2.20	6.26±2.40	65±15	4.44±2.54
	8	2	3.83±1.18	6.54±0.64	90±10	5.82±0.08
9000	4	2	0	0	0	0
	5	2	0	0	0	0
	6	2	0.81±0.26	1.38±0.16	65±15	0.92±0.30
	7	2	1.59±0.55	2.69±0.37	73.33±6.67	1.95±0.09
	8	2	3.11±0.93	5.32±0.46	70±10	3.68±0.21

Experiments were conducted using 2 different females as replications
RC* Relative to control after adjustment of the latter to 100%.

5.3.2 Optimization of cold shock

The preliminary results of the experiments conducted using cold shock had a maximum triploid rate (100%) at 2°C and timings of application of 20 min duration shocks. The later experiments were set up to maximize the effects of cold shock on survival rates at different stages of larval development and to optimize the intensities, timings of application and duration of cold shocking at a fixed temperature of 2°C. The full list of experiments conducted and results obtained is shown in Table 5.4. The mean survival rates at different development stages of embryos showing the effects of cold shock at

2°C are presented in a more convenient format in Figure 5.4. All levels of 2°C cold shock were most effective to induce triploidy and survival of larvae when given in the period of 3-6 min TAF and 15-30 min durations. The treatment of 5 min TAF and 20 min duration give the highest survival to hatch out of embryos at 66.56±2.85 %, while the treatment of 4 min TAF and 15 min duration give the maximum rate of viable 3 day old fry at 63.85±0.10 % relative to control diploid hybrids. All of these applications of cold shock resulted in 100% triploid rates, confirmed by chromosome counts. The optimum timing identified for treating fertilized eggs for 15 min duration to induce high survival rate and high triploid rate at most effective temperature of 2°C was 4 min TAF. Later timings of administration of cold shock (5-6 min TAF) resulted in a generally lowered survival rate of embryos. The treatment administered at 3 min TAF gave generally lowered survival rate as well as higher rates of deformed bodies of embryos. At later TAF (5-6 min), survival rate was once again reduced but in this case, it was a consequence of both abnormality and fewer embryos surviving the treatments. The intermediate timing administered at 4 min TAF of 2°C cold shock for 15 min duration was found to be close to the treatment optima in inducing 100 % triploid rate. Cold shock treatment of 6 min TAF and 30 min duration gave very low survival to hatch and 0 % of embryo servived to 3 days of age (Table 5.4).

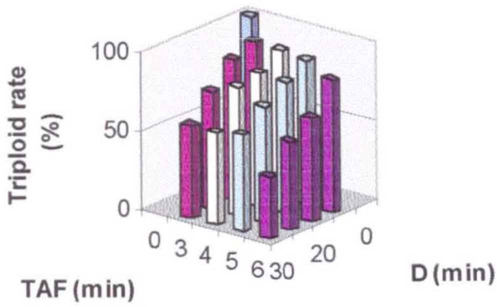
The ploidy of all treatment and control batches was determined by chromosome preparation from a subsample of newly hatched or one day old larvae. The triploid big-oui hybrid metaphases are composed of three sets of chromosome (3n=82). Although it was difficult to count the number of chromosomes exactly, the easily identified by the presence of three sets of chromosome which high complexity were of individual

chromosome spreads (Figure 5.5A). The triploid rate was determined by the karyological analysis of several (more than 3) karyotypes per individual larvae and 20 individuals per treatment. The results were expressed as mean triploid rates which were combined with survival rates for determination of the optimum treatment parameters in inducing triploidy.

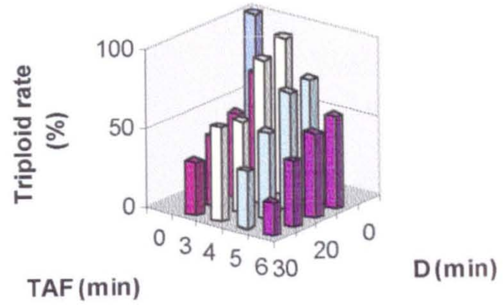
Erythrocyte nuclear measurements were also used for determination of ploidy levels in this study. Blood samples were drawn from the caudal vein of 20 individual catfish species of grow-out experiment. Blood smears were prepared and stained with Wright's stain, fields of erythrocytes were photographed and negatives were projected with an enlarger to give a final magnification of x1000. A sample photograph of diploid and triploid erythrocyte was shown in Figure 5.5B.

5.3.3 Comparative studied on grow out of diploid, triploid big-oui hybrid and pure species

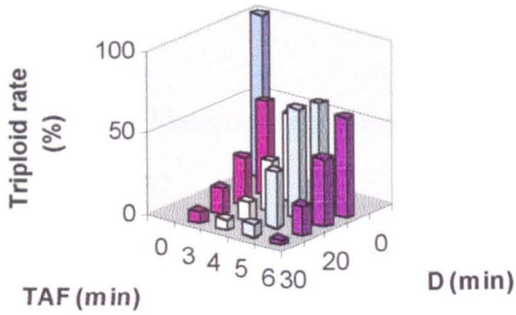
The comparative data for growth experiments on diploid, triploid and pure species, conducted in 8 glass aquaria (2 replications) for 184 days showing the results in detail by mean survival rate, mean initial length and weight, mean final length and weight and specific growth rate (SGR) are given in Table 5.5 and Table 5.6. The pooled data for mean weight gain and mean length gain of 4 groups of catfish are shown in Table 5.7-5.8andFigure5.6A-Brespectively.



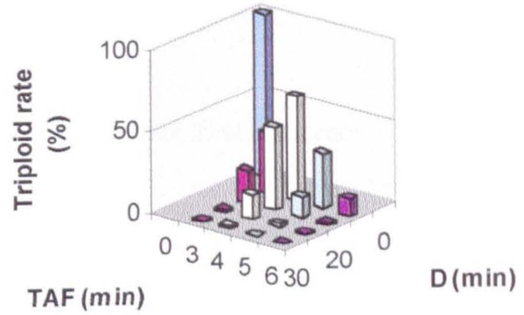
(a)



(b)



(c)



(d)

Figure 5.4 Survival rate of triploid hybrid catfish at difference development stage from cold shock experiment at 2° C

a. fertilisation

b. somite stage

c. hatch out

d. 3 days of age

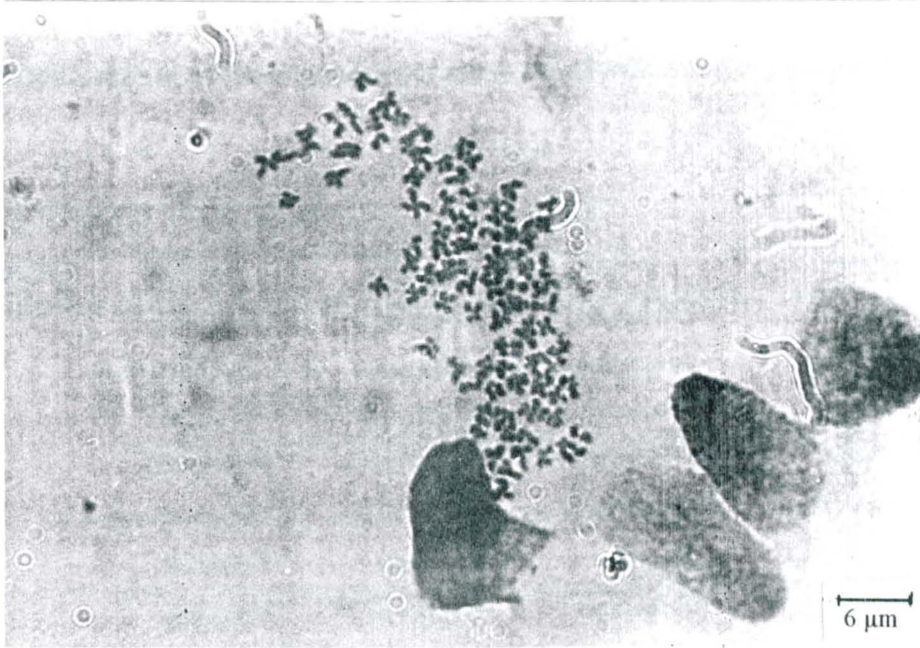


Figure 5.5A Metaphase chromosomes of triploid big-oui hybrid, chromosome number 3n=82

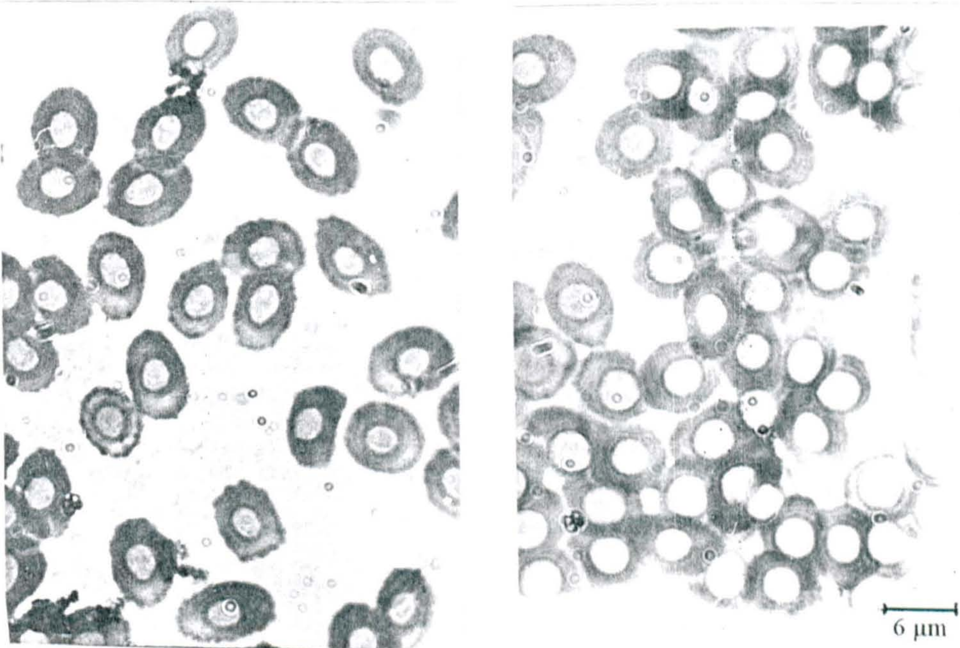


Figure 5.5B Erythrocytes of diploid and triploid big-oui hybrid

Table 5.4 Mean fertilisation and survival rate (relative to control) at different development stage of triploid hybrid embryos from cold shocking experiment at 2°C

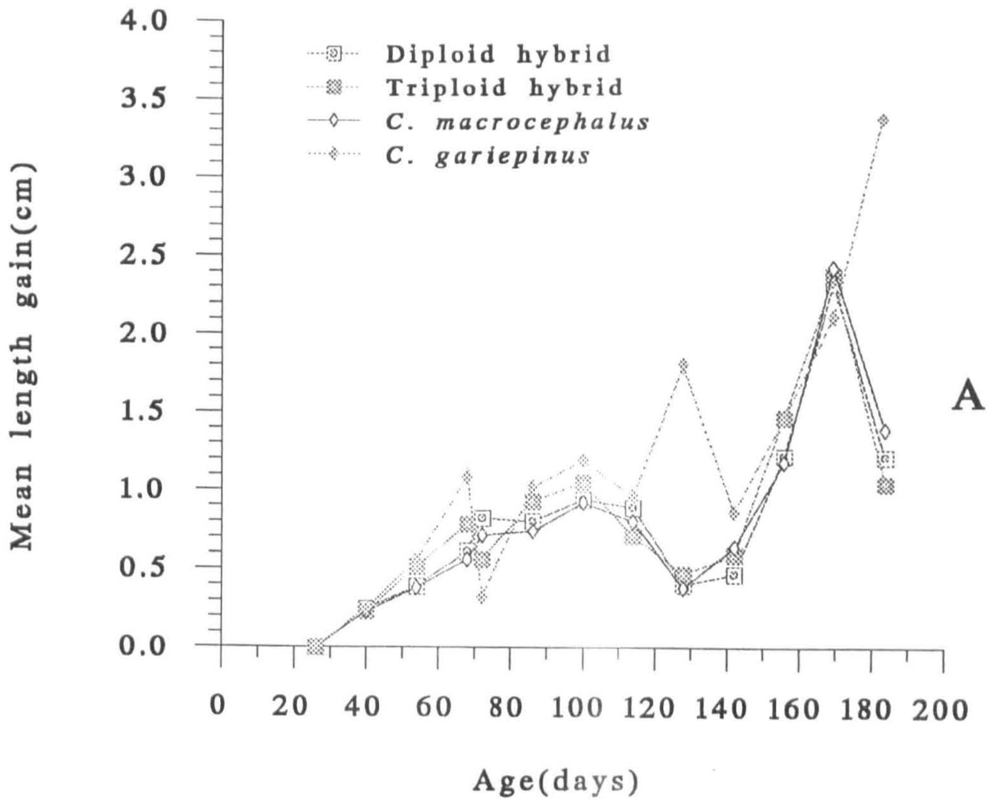
Treatment	%±SE			
TAF(min)/D(min)	Fertilisation	Somite stage	Hatch out	3 days of age
0/0(cont)	100.00 ± 0.00^{g}	100.00 ± 0.00^{e}	100.00 ± 0.00^{i}	100.00 ± 0.00^{i}
(Actual)	(78.86±8.01)	(61.50±10.71)	(42.14±7.91)	(32.25±6.94)
3/15	93.80 ± 0.17^{f}	72.75 ± 0.76^{abcd}	56.29 ± 1.63^{fgh}	36.57 ± 2.76^{fg}
3/20	88.54 ± 0.88^{ef}	52.66 ± 1.26^{abc}	28.21 ± 0.28^{bcdef}	19.36 ± 1.78^{def}
3/25	74.00 ± 0.19^{cd}	43.53 ± 1.29^{ab}	15.15 ± 0.02^{abcd}	1.30 ± 0.59^{ab}
3/30	58.22 ± 0.46^{abc}	33.09 ± 0.23^{ab}	6.69 ± 0.10^{ab}	0.36 ± 0.36^{a}
4/15	90.43 ± 0.11^{ef}	99.43 ± 16.69^{de}	53.29 ± 1.24^{fgh}	63.85 ± 0.10^{h}
4/20	84.65 ± 0.18^{def}	91.17 ± 17.16^{cde}	30.63 ± 0.43^{bcdefg}	50.33 ± 1.65^{gh}
4/25	81.60 ± 0.34^{de}	58.16 ± 7.47^{abc}	11.70 ± 0.35^{abc}	15.06 ± 1.40^{de}
4/30	58.27 ± 1.13^{abc}	59.62 ± 0.42^{abc}	6.47 ± 0.21^{ab}	1.98 ± 0.72^{abc}
5/15	91.88 ± 0.23^{ef}	77.42±0.25bcde	64.36 ± 7.15^{h}	33.16 ± 0.85^{efg}
5/20	83.35 ± 0.19^{de}	74.82 ± 0.66^{bcd}	66.56 ± 2.85^{efgh}	12.67 ± 0.23^{cd}
5/25	73.79 ± 0.17^{cd}	55.12 ± 0.68^{abc}	34.44 ± 4.19^{cdefgh}	1.99 ± 0.28^{abc}
5/30	61.80 ± 0.59^{bc}	36.79 ± 0.11^{ab}	8.46 ± 0.36^{abc}	0.00 ± 0.00^{a}
6/15	83.66 ± 0.24^{de}	58.16 ± 0.08^{abc}	60.11 ± 2.00^{gh}	10.43 ± 0.15^{bcd}
6/20	65.54 ± 0.70^{bc}	53.07 ± 0.44^{abc}	40.95 ± 4.56^{defgh}	1.59 ± 0.23^{ab}
6/25	55.88 ± 0.66^{ab}	41.34 ± 0.60^{ab}	18.01 ± 1.76^{abcde}	0.63 ± 0.09^{a}
6/30	39.65 ± 0.16^{a}	20.95 ± 0.29^{a}	3.08 ± 0.01^{a}	0.00 ± 0.00^{a}
±SEM	0.02	0.20	0.08	0.04

Data in the same column carrying different superscripts denote a significant difference by ANOVA at $p<0.05$ between values in each development stage.

At stocking (26 days of age), the mean initial weight of fish belonging to diploid, triploid and pure *C. macrocephalus* groups was not significantly different, although there were differences between pure *C. gariepinus* and the other 3 groups of fish. The mean initial length of fish belonging to diploid and triploid groups was not significantly different, while there were significantly differences between these 2 groups of hybrids, pure *C. macrocephalus* and pure *C. gariepinus*. At the end of experiments (184 days of age), the mean final length, mean final weight and SGR were not significantly different in the fish belonging to diploid, triploid hybrid and pure *C. macrocephalus* groups: only the fish in the pure *C. gariepinus* group were significantly different to the other groups.

During the whole on-growing period of 6 months, the pure *C. gariepinus* group grew faster compared to the other groups, while it had a lower survival rate than the other groups. However the growth rate (both weight and length comparisions) of diploid and triploid big-oui hybrid were not significantly different at $p<0.05$ (Table 5.6).

At the end of the on-growing experiment, all of the fish in each group were measured and weighed and the length-weight relationship of each groups were calculated. The linear regression lines and equations of 4 groups of catfish are shown in Figures 5.7-5.10.



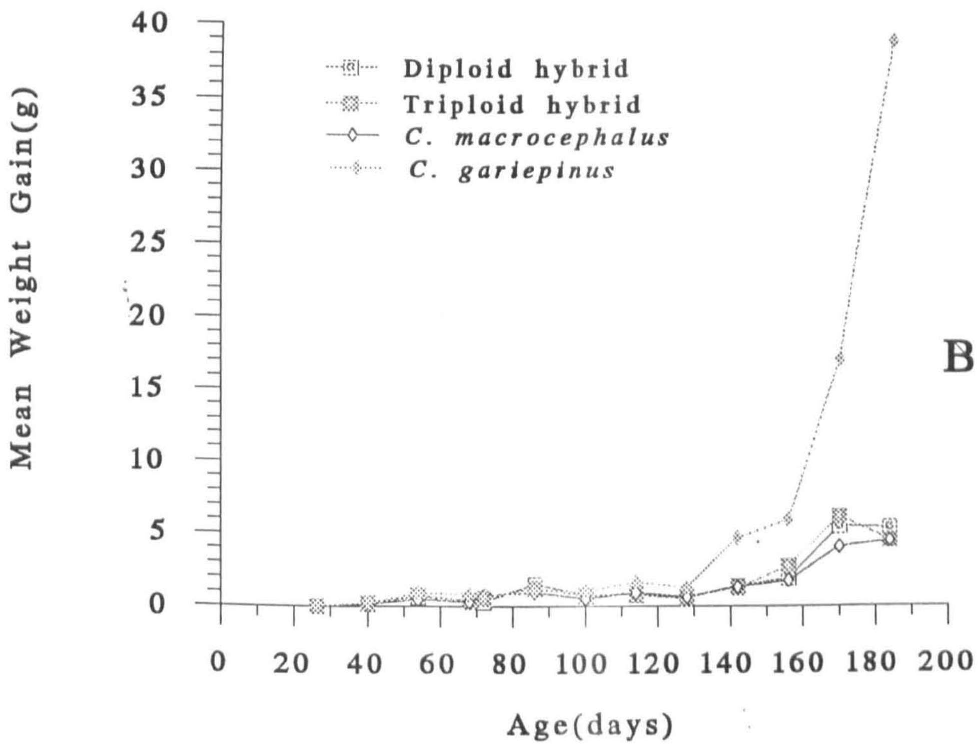


Figure 5.6 Comparison of growth in 4 groups of catfish (A) Mean length gain; (B) Mean weight gain

Table 5.5 Mean survival rate of diploid hybrid, triploid hybrid and pure species on grow out experiment

Age (days)	Diploid	Triploid	*C. macrocephalus*	*C. gariepinus*	±S.E.M
26	100.0±0.00^{a}	100±0.00^{a}	100.0±0.0^{a}	100.0±0.0^{a}	0.00
40	89.5±3.5^{a}	85.0±3.0^{a}	92.0±2.0^{a}	89.0±3.0^{a}	1.46
54	78.5±1.5^{a}	74.5±0.5^{a}	86.0±3.0^{a}	78.5±6.5^{a}	1.83
68	74.5±10.5^{a}	71.0±1.0^{a}	81.5±0.5^{a}	68.5±5.5^{a}	2.98
72	71.0±8.0^{a}	68.0±2.0^{a}	79.0±1.0^{a}	63.5±4.5^{a}	2.36
86	64.0±8.0^{a}	65.0±4.0^{a}	78.5±0.5^{a}	57.5±4.5^{a}	2.51
100	61.0±9.0^{ab}	57.0±3.0^{ab}	73.0±1.0^{b}	49.5±2.5^{a}	2.47
114	55.0±10.0^{ab}	51.0±1.0^{ab}	64.0±4.0^{b}	37.0±2.0^{a}	2.75
128	50.5±7.5^{ab}	46.5±2.5^{ab}	62.5±3.5^{b}	35.0±2.0^{a}	2.22
142	45.5±6.5^{b}	43.0±1.0^{b}	59.0±6.0^{b}	22.00±1.0^{a}	2.24
156	42.0±4.0^{b}	39.0±1.0^{b}	57.0±6.0^{c}	18.5±1.5^{a}	1.86
170	34.5±5.5^{b}	37.5±1.5^{bc}	56.0±2.0^{c}	16.0±3.0^{a}	1.69
184	34.0±5.0^{b}	33.0±1.0b	46.5±5.5^{b}	15.0±3.0^{a}	2.02

Mean survival rate at the same age (days) carrying the superscripts denote statistically significant difference by ANOVA at $p \leq 0.05$ between the value in each species.

5.3.4 Effects of triploidy on sexual development

Data of average gonad weight and gonado-somatic index (GSI) together with mean standard length and body weight for male and female in the four groups at the end of the on-growing experiment (6 months) are presented in Table 5.7. At 6 months of age, the GSI's of both the diploid and triploid hybrids in both sexes were lower than in the pure species, while comparision between diploid and triploid hybrids shared relatively little difference for both sexes.

5.3.5 Gonadal histology of diploid, triploid hybrid and pure species.

Histological sections of diploid and triploid hybrid ovaries at 6 month of age contained mainly oogonia and a few small primary or previtellogenic oocytes. Most of the cells were of similar size with very few undergoing divisions, some of the primary previtellogenic oocytes started to show retarded development. In most of histological slides, very few secondary vitellogenic oocytes were found in triploid ovaries or in diploid hybrids at the same age (Figure 5.11A and 5.11B).

Histological sections of both pure *C. macrocephalus* and pure *C. gariepinus* ovaries at 6 months of age had strongly basophilic cytoplasm and lightly stained round nuclei in the developing primary and secondary previtellogenic oocytes. Some ovaries sampled contained oogonia and maturing vitellogenic oocytes with regular nuclei and vacuolated cytoplasm which is associated with endogenous yolk formation (Figure 5.12A and 5.12B).

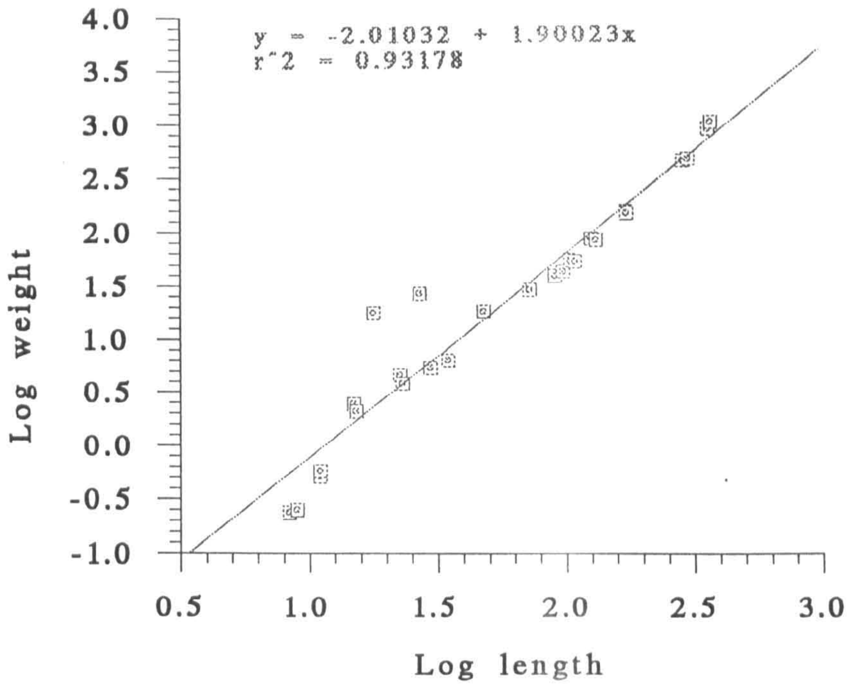


Figure 5.7 Length-weight relationship of diploid hybrid catfish

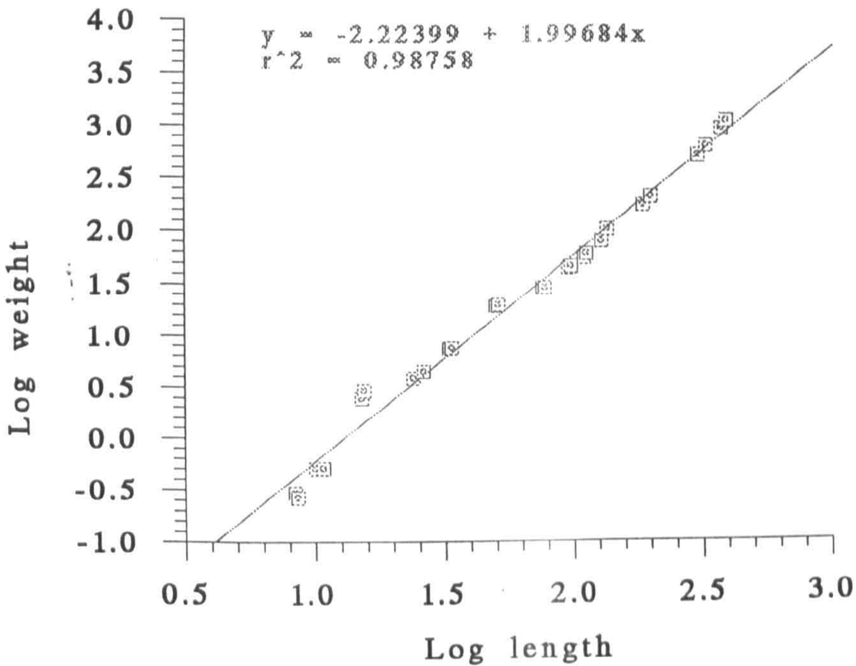


Figure 5.8 Length-weight relationship of triploid hybrid catfish

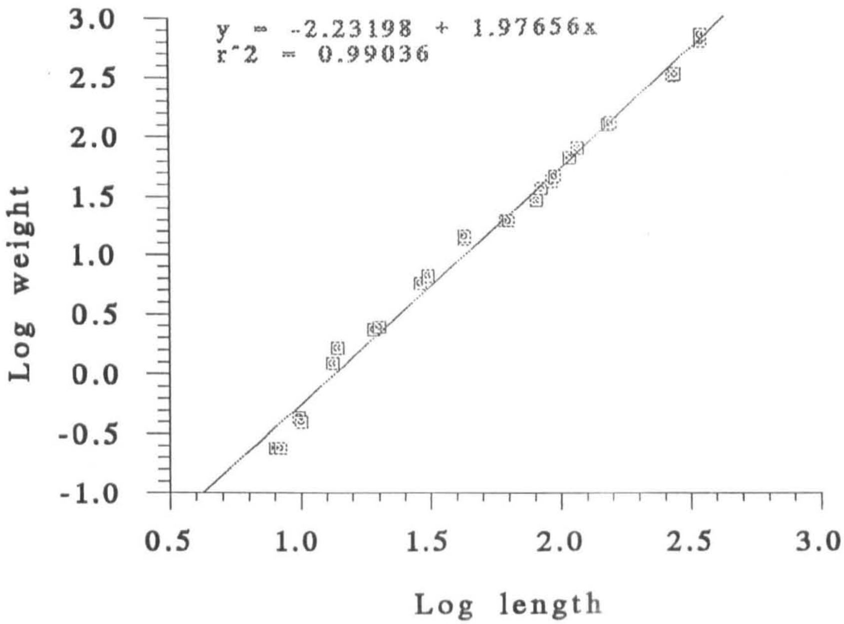


Figure 5.9 Length-weight relationship of *C. macrocephalus*

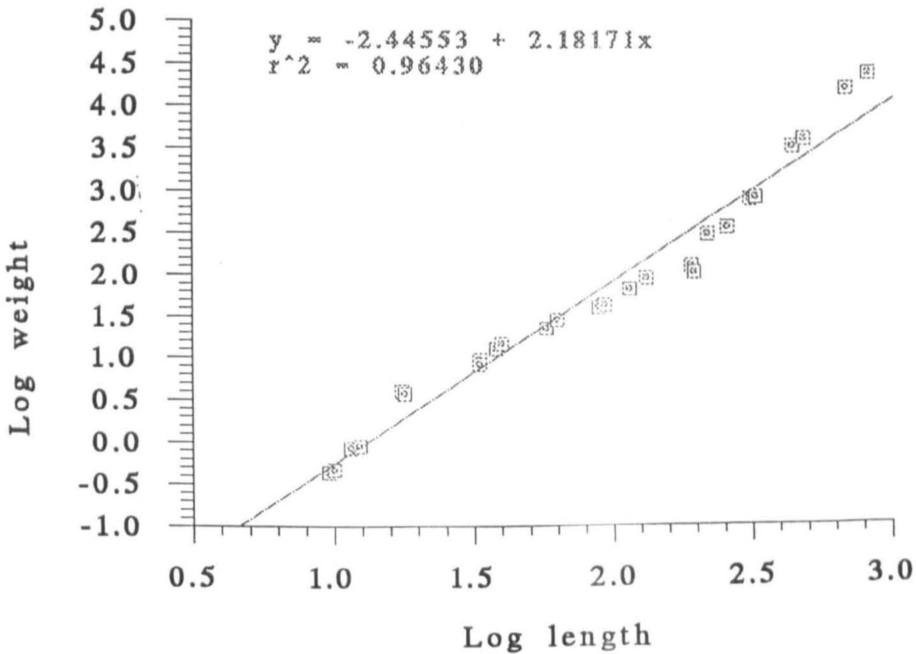
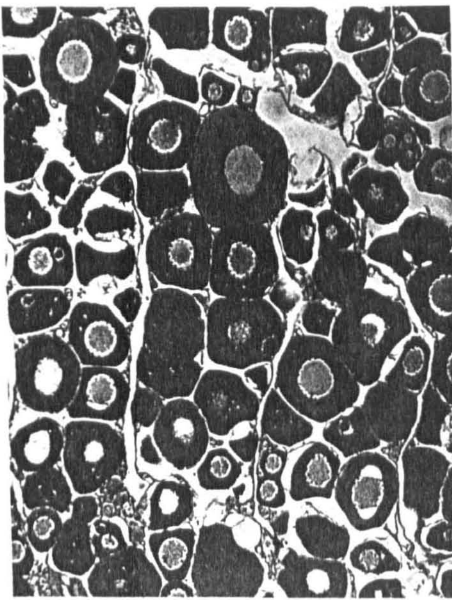
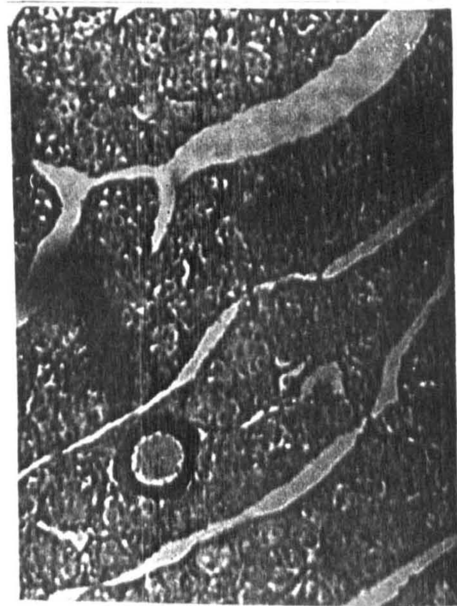


Figure 5.10 Length-weight relationship of *C. gariepinus*

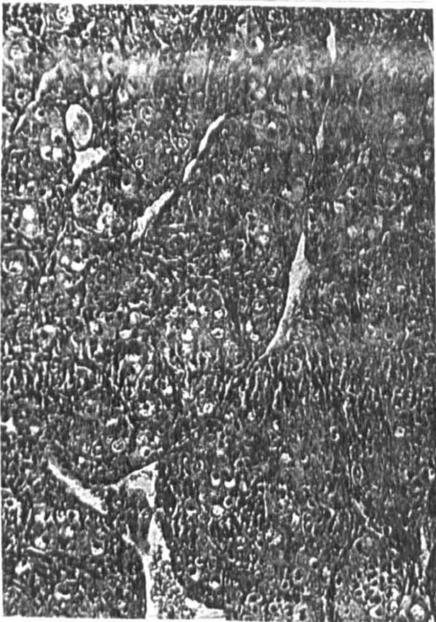


A

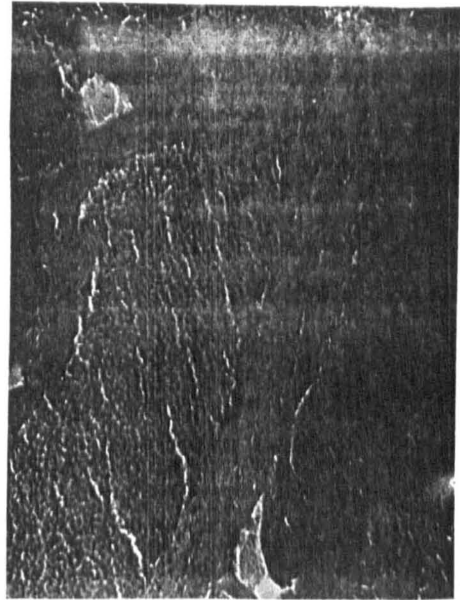


B

156 μm



C



D

Figure 5.11 Histological section of gonad in diploid and triploid big-oui hybrid at 6 months of age.

A:diploid ovary

B:triploid ovary

C:diploid testis

D:triploid testis

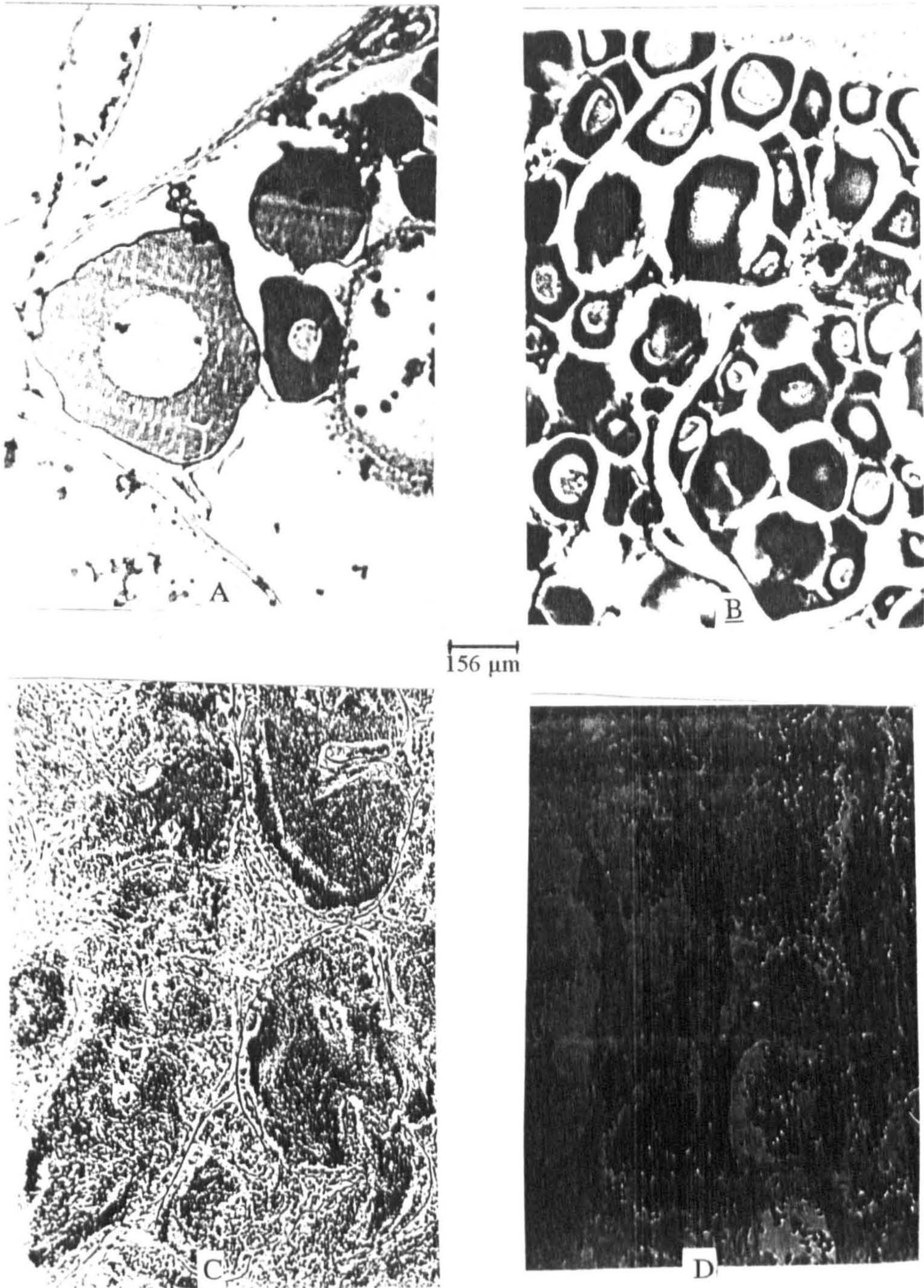


Figure 5.12 Histological section of gonads in pure species at 6 months of age.

A:*C. macrocephalus* ovary

B:*C. gariepinus* ovary

C:*C. macrocephalus* testis

D:*C. gariepinus* testis

Table 5.6 Comparision on growth rate of diploid, triploid big-oui hybrid and the pure species

Measurements	Diploid hybrid	Triploid hybrid	Pure *C.macrocephalus*	Pure *C.gariepinus*	±SEM
Mean initial length	2.55 ± 0.05^{ab}	2.53 ± 0.01^{ab}	2.49 ± 0.03^{a}	2.69 ± 0.03^{b}	0.02
Mean initial weight	0.55 ± 0.01^{a}	0.58 ± 0.02^{a}	0.54 ± 0.00^{a}	0.71 ± 0.01^{b}	0.01
Mean final length	13.00 ± 0.09^{a}	13.20 ± 0.17^{a}	12.81 ± 0.06^{a}	17.66±0.77b	0.20
Mean final weight	19.66 ± 0.56^{a}	20.49 ± 0.73^{a}	16.75 ± 0.41^{a}	74.66 ± 6.46^{b}	1.63
SGR (%)	2.27 ± 0.02^{a}	2.26 ± 0.04^{a}	2.17 ± 0.02^{a}	2.94 ± 0.06^{b}	0.02

Data in the same row carrying the superscript denote a significant difference by ANOVA at $p \leq 0.05$ between values in each characteristic measurement

Table 5.7 Mean body weight, gonadal weight and gonadosomatic index (%GSI) of male and female in 4 groups of catfish in grow out experiment at 184 days of age.

Species	Sex	No.of fish	Mean body weight	Mean gonad weight	Mean GSI (%)
2n hybrid	male	25	20.60±5.37	0.06±0.04	0.29±0.15
	female	25	20.34±5.39	0.12±0.09	0.55±0.29
3n hybrid	male	22	19.87±5.72	0.07±0.07	0.30±0.26
	female	28	19.51±4.18	0.18±0.55	0.37±0.20
C.macrocephalus	male	24	15.94±3.65	0.07±0.04	0.39±0.15
	female	26	17.50±4.74	0.23±0.11	1.26±0.30
C.gariepinus	male	14	69.66±48.61	0.64±0.54	0.84±0.34
	female	16	76.61±41.85	1.71±1.16	1.90±0.74

% GSI = Mean gonad weight / Mean body weight x 100

Histological sections of diploid and triploid hybrid testes at 6 months of age contained very few cysts with spermatogonia and spermatocytes in which the cells were under going active divisions and had developed into spermatozoa. In most of the testicular sections of triploid hybrids, there were very few primary sperm cells resulting in nearly empty tubules having lightly stained seminal fluid containing only a few spermatozoa (Figure 5.11C and 5.11D).

The histological sections of pure species showed they contained highly distinct cysts surrounded by the basal lamina at all stages of development. There were

spermatogonia, spermatocytes, spermatids and a large number of spermatozoa (Figure 5.12C and 5.12D).

5.3.6 Gonadal condition and reproduction of triploid big-oui hybrid

In this study, some of triploid big-oui hybrids and diploid hybrids (control) produced from optimizing cold shock experiments were kept for over one year of age until they reached (expected) maturation stage. The gonadal condition in 10 individuals of both sexes in each group of fish were examined and compared the size and weight. The comparision between triploid and diploid hybrid GSI of matured fish is shown in Table 5.8. It was observed that diploid female ovaries were 5-10 times bigger than triploid ovaries and packed with numerous developing oocytes, while triploid ovaries of the same age of fish were very small compared to the body size, without developing oocytes. The ovaries of triploid fish contain mainly oogonia. When triploid females have reached an age of six months, follicular atresia of the previtellogenic oocytes occur (Figure 5.11). Diploid male testes were soft, elongate, unpairing lobe, clear brown-red coloured and with few spermatozoa. Triploid male testes were usually a little smaller consisted of harder tissue, more white in colour and without spermatozoa and when the males have reached the age of six months, the primary spermatocytes start to degenerate, and the cysts start to fuse, giving rise to degenerating tubules (Figure 5.11 and 5.13). Morphologically, triploid males showed similar development of secondary sexual characters, having the pointed prominent urogenital papilla as in mature diploid hybrid males.

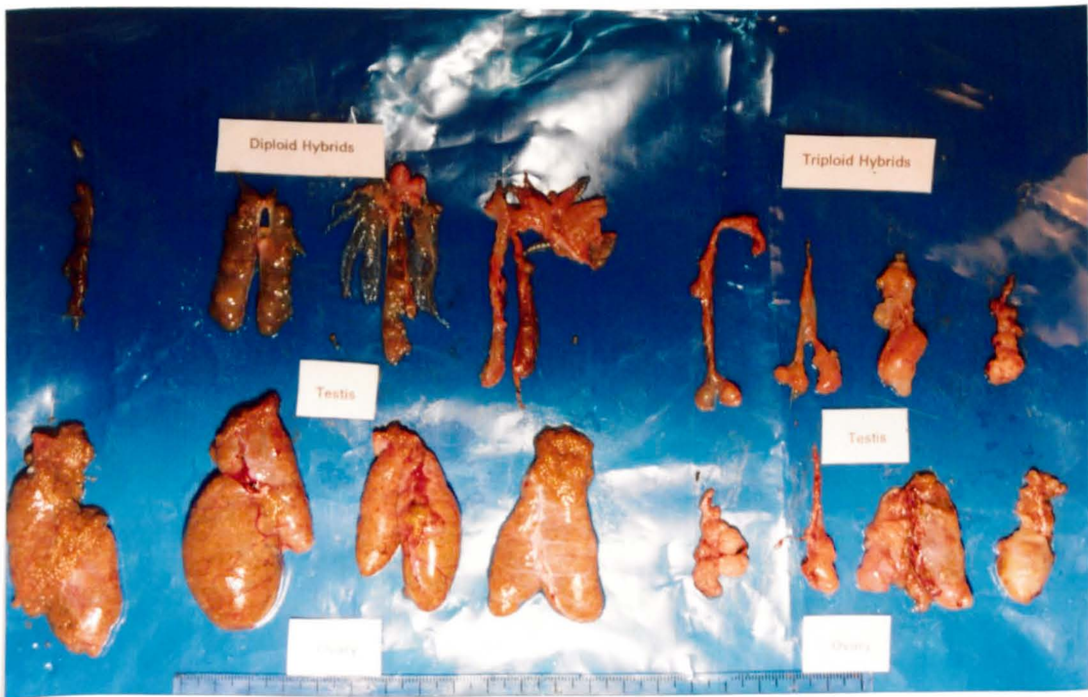


Figure 5.13 Morphological different of gonad in mature diploid and triploid big-oui hybrid at the age >1½ years.

Table 5.8 Mean standard lenght, body weight, gonad weight and GSI of mature diploid and triploid hybrid catfish

Species	Sex	No.of fish	Body length (cm)	Body weight (g)	Gonad weight (g)	GSI (%)
2n hybrid	male	10	37.69±0.98	795.50±65.97	6.88±2.53	0.87±0.34
	female	10	37.17±1.65	940.50± 178.04	18.12±3.18	1.96±0.39
3n hybrid	male	8	36.14±2.24	700.00±87.05	5.33±2.28	0.74±0.27
	female	10	36.77±1.40	812.00±63.52	9.86±3.55	1.21±0.42

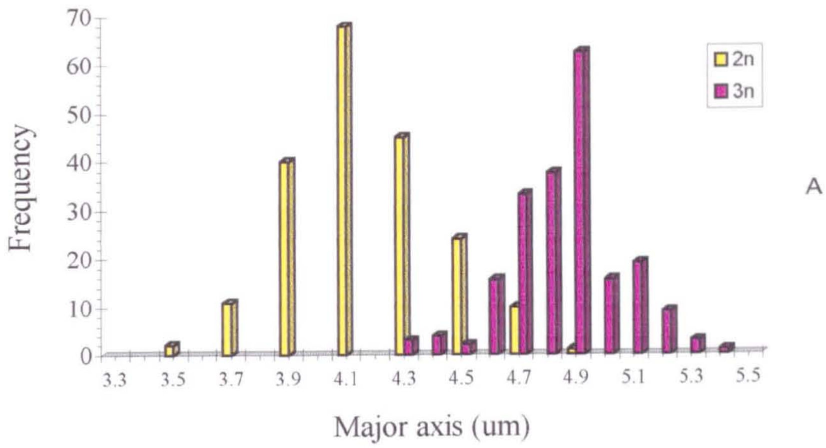
5.3.7 Erythrocyte nuclear measurements of diploid, triploid big-oui hybrid and pure species

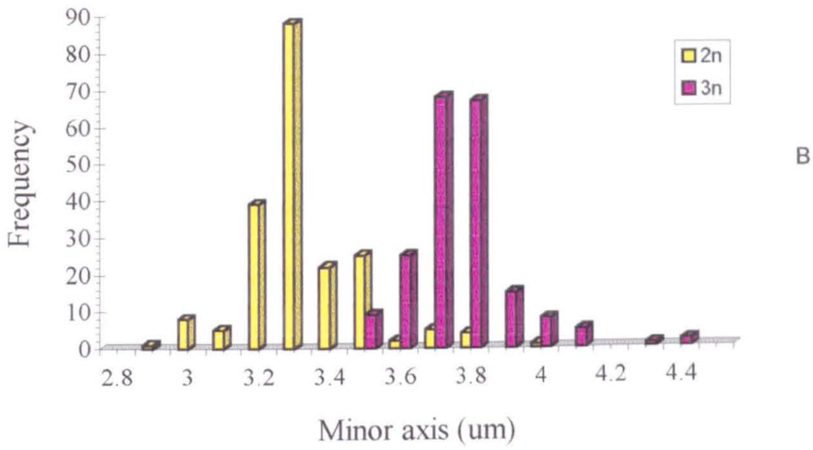
Minor axis, major axis and volume measurements of erythrocyte nuclei in 4 groups of catfish from grow-out experiments were compared by ANOVA to determine which variable best predicted ploidy levels. The average values of major axis, minor axis and erythrocyte nuclear volume of each were calculated (Table 5.9) and the frequency distribution of erythrocyte nuclear measurements of diploid and triploid big-oui hybrid were compared (Figure 5.14). Diploid hybrid, *C. gariepinus* and *C. macrocephalus* have an average major axis, minor axis and nuclear volume not significant different while only triploid hybrid has significant different with other groups of fish. The nuclear volume of diploid hybrids is nearly 2/3 of that of triploid hybrids.

Table 5.9 Mean erythrocyte nuclear measurements for diploid, triploid big-oui hybrid and pure parental species.

Species	Variable		
	Major axis(μm)	Minor axis(μm)	Volume(μm^3)
Diploid Hybrid	4.15 ± 0.25^a	3.33 ± 0.16^a	24.24 ± 2.93^a
Triploid Hybrid	4.85 ± 0.18^b	3.74 ± 0.14^b	35.78 ± 3.43^b
C. gariepinus	4.15 ± 0.16^a	3.31 ± 0.08^a	23.94 ± 1.59^a
C. macocephalus	4.12 ± 0.16^a	3.34 ± 0.09^a	24.11 ± 1.81^a

Different superscripts denote a significant difference by ANOVA at $p<0.05$ between values in the same column.





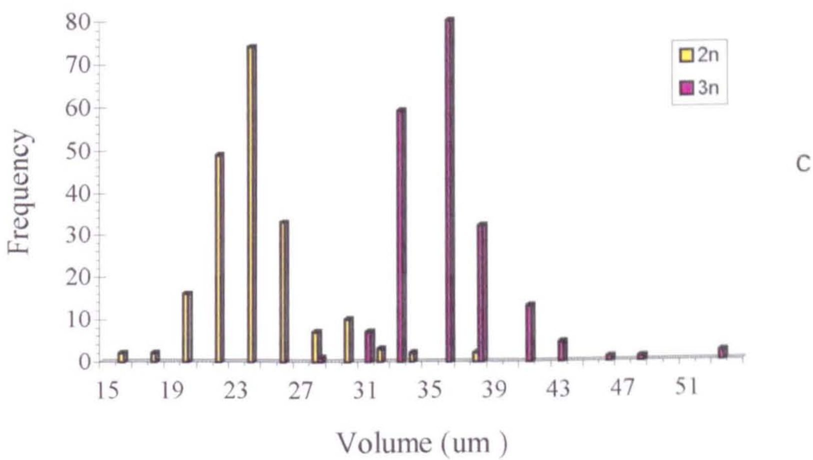


Figure 5.14 Frequency distribution of erythrocyte nuclear measurements of diploid and triploid big-oui: (A) Major axis (B) Minor axis (C) Volume.

5.4 Discussion

5.4.1 Optimisation of treatment parameters for triploidy induction

The results of cold shock treatments performed to induce triploidy are consistent with the hypothesis that during the 2nd meiosis, the spindle apparatus of fertilized eggs is susceptible to disturbance in a very short period of time, approximately 1-2 min, between 4-5 min after fertilisation at 27±1°C. Later treatments, although allowing some survival, are progressively less effective in inducing triploidy because the 2nd meiotic division has already been completed. Earlier treatments of cold shock resulted in no triploidy induction and caused lower survival of embryos, possibly because improper timing and deterious effects of the treatments resulted in the production of inviable aneuploids. Cold shock intensities over or below the optimum resulted in incomplete retention of the 2nd polar body which produced aneuploidy, deformed and killed more embryos and also reduced triploid rates. Longer or shorter cold shock durations than the optimum also reduced the triploid rates and yields due to a drastic effect on survival rate.

From the above discussion it is clear that cold shock treatments can be deleterious if the conditions are sub-optimal. Chourrout (1984) observed a high frequency of abnormal aneuploid embryos caused by incomplete retention of the second polar body at the low shock intensities in rainbow trout. Therefore, treatment optima can be considered as

residing on a plateau, on the slopes of which the triploid yield is reduced due to a drastic effect on survival rate and/ or triploid rate.

In this study, the results of optimisation of cold shock treatments are comprehensive and slightly different from previous works on cold shock induction of triploidy in *C. gariepinus* (Richter *et al.*,1987), in *C. macrocephalus* (Vejaratpimol and Pewnim, 1990), and in *Clarias batrachus* (Manickam, 1991). Previous workers used only one temperature (5°C), fixed 40 min duration, 2-4 min TAF (Richter et al.,1987), various 5, 10, 15, 30, 45, and 60 min D, fixed 2 min TAF (Vejaratpimol and Pewnim, 1990) and fixed 1.0 h duration, fixed 5 min TAF (Manickam, 1991) of application of shock. These shock temperatures gave poor results in the present experiments. Less viable triploidy could be induced because of greater lethality and deleterious effect of cold shock on embryonic survival of this particular shock intensity. This study further revealed that colder shock temperature (2°C) induces 100 % triploidy without an increased number of deformed embryos.

The cold shock (2°C) administered for 15-20 min duration at 4 min TAF was most effective in inducing 100 % triploidy in big-oui hybrid. This indicates that pressure shock treatments might be species specific. The temperature for application of cold shocks on fertilised eggs are not consistent with other studies, because the present findings in big-oui hybrid revealed that any cold shock at lower than 5°C resulted in induced triploidy and few deformed embryos. On the other hand, 5°C cold shock intensity was effective and reliable in inducing triploidy but gave lower larval survival rates. The only other report on ploidy manipulation using cold shock in *Clarias* catfish is a study on feed utilization between diploid and triploid African catfish by Henken *et*

al. (1987). They reported that triploid fish were obtained by cold-shocking at 5°C for 40 min starting 3 mins after fertilisation. This dose produced about 95 % triploidy. This is lower than those shown to be maximally efective in the present study for inducing triploidy. Although it was shown earlier that this temperature of cold shock is effective, when of sufficient duration to induce triploidy by itself, the duration of 40 mins shock used by Henken *et al.* (1987) and Richter *et al.* (1987) is longer than that reported by this study to be maximally effective. This may mean that the difference in cold temperature ranges reported by those studies are the result of the administering time and cold shock acting synergistically for interference with the meiotic event.

From the present results it appears that the most effective heat shocks must be administered in the same TAF period of the most effective cold shocks (5 min). It could be concluded at the time of the second meiosis in this species is about 4-5 min after fertilisation. The results from this study observed a high frequency of abnormal aneuploid embryos caused by incomplete retention of the second polar body at the low intensities of heat shocks during the induction of triploidy in big-oui hybrid. In a detailed experiment on the effect of sub-optimal heat shock on mortality and deformity in young fish, Elahi (1990) reported that sub-optimal shock durations, time after fertilisation and low intensity produced higher rates of aneuploidy, lower survival and fewer triploids. Shock intensities between 38-39°C were found to be optimal in the present trials and shorter shock durations performed poorly in inducing triploidy.

The pressure shock in this study was less effective as an agent for triploidy induction. It was observed to be less ideal, even lethal, compared to cold and heat shock, resulting in high variations in survival rates of embryos and low effectiveness in inducing triploidy.

On the basis of the previous studies a conclusion could be drawn that pressure shocks, as they cause a suppression of the role of spindle fiber of the meiotic division events, have to be applied at a relatively later time after insemination than are necessary for either cold or heat shock but must be applied at the optimum duration and intensities, in order to be effective. The present results suggest that the timing of initiation of optimum cold shock should be slightly earlier than heat shock and pressure shock. This may indicate that these agents have different lag times before exerting their effects.

Confirmation of triploid induction by karyotyping and erythrocyte nuclear measurement compared with diploid hybrids showed that the chromosome number of the triploid hybrid is 3n=82 while the diploid hybrid has 2n=55 and the mean erythrocyte nuclear volume of diploid hybrid is nearly 2/3 of that of triploid hybrid. Erythrocyte nuclear volumes have been widely cited as well established means for the determination of ploidy because an increase in the number of chromosome sets causes a proportional increase in the size of the cell nucleus. Theoretically a change in chromosome set from a diploid state to a triploid would result in a 1/3 increase in nuclear size. The mean values for erythrocyte nuclear volumes in this study closely agree with the predicted increase. Triploid big-oui hybrid erythrocyte nuclei were 1.48 times larger than diploid hybrid nuclei. Previous studies using nuclear volumes have

found similar results for fish of known ploidy (Swarup, 1959; Purdom, 1972; Wolters, *et al.*, 1982b).

5.4.2 Performance of growth in diploid and triploid big-oui hybrid.

In the context of the present study, although the overall growth performance of triploids big-oui hybrid was not significantly better than the diploids, it can be concluded that triploid big-oui hybrid might show better performance under natural pond conditions as opposed to laboratory aquaria. The reason is that in natural condition diploid big-oui hybrid have fertile gonads, while sterile triploid hybrids may devote the energy that would otherwise go into the production of large volumes of eggs into increased growth.

5.4.3 Gonadal development and sexual maturation in diploid and triploid big-oui hybrid

The main aim of inducing triploidy in fish was to develop a sterile population in an attempt to prevent precocious sexual maturation and fertility. Early maturation processes in diploid fish, particularly females, often have profound and ultimately limiting effects on growth resulting from lossing of energy that would go into the production of large volumes of eggs. In conclusion, the present study can be summarised with the hypothesis that triploid male and females are functionally and endocrinologically sterile because their germ cells can not continue meiosis (Ihssen, *et al.*, 1990). In triploid females, their ovaries might produce less steroidogenic tissue and less hormone, therefore, they exhibit none of the endocrine changes relative to normal sexual maturation and ultimately no success in functional oocyte development (Billard,

1989). Triploid males are functionally sterile and not able to produce spermatids leading to aneuploid spermatozoa as same as Richter, *et al.*, (1987) whose reported that the testes of triploid fish contain cysts with spermatogonia and cysts with primary spermatocytes, which blocked in prophase 1 of meiosis.

CHAPTER SIX

GYNOGENETIC DIPLOID INDUCTION IN WALKING CATFISH *C. MACROCEPHALUS* USING SPERM OF THE AFRICAN SHARPTOOTH CATFISH *C. GARIEPINUS*

6.1 Introduction

In animals, meiosis in the egg is the principal cell division phase where manipulation is possible, and in fish and other animals with external fertilization, artificial processes can be applied to either gamete before fertilization or to the fertilized eggs at any period during the formation of the zygote. Two basic fields of practical importance involve the processes of parthenogenesis and induced polyploidy, respectively (Purdom, 1983). Gynogenesis is accomplished by initiating the second meiotic division in the egg by fertilizing the egg with DNA-deactivated sperm. DNA-deactivated sperm are produced by irradiating the milt with ultraviolet light (UV light) or gamma radiation until all the nuclear DNA is denatured but the sperm is still motile. If the fertilized egg is shocked prior to the extrusion of the second polar body or at first cleavage then this will be retained within the egg and effectively made diploid. The egg shocked at the time of the second meiotic division (as for triploidy induction) produces a "meiotic gynogenome". Alternatively, it can be shocked to interfere with the first cell division (as for tetraploid induction), producing a "mitotic gynogenome" (Hussain, *et al.*, 1991).

The genomes of meiotic and mitotic gynogenetics are different. Meiotic gynogenetics are highly inbred (approximately 50 %) particularly for genes near the centromeres of the chromosomes. This variation results from the reassortment of genes during recombination. Mitotic gynogenetics are 100 % homozygous

because they are produced by the replication of chromosomes at mitosis. Androgenesis has been also successfully induced in fish by irradiation of ova, fertilisation of eggs with normal sperm, and suppression of the first mitosis with high pressure or temperature treatments (Ihssen *et al.*, 1990). Gynogenetic diploids have been used for cytogenetic studies of meiotic phenomena and gene mapping.

At present, gynogenesis has more applications in aquaculture research than aquaculture production. Diploid gynogenesis has been used to investigate sex determination in fish. By artificial gynogenesis fish produced carrying two sets of maternal chromosomes with no paternal genetic contribution will be monosex all-female populations if sex inheritance of the species in question is of female homogametic type (Suzuki, *et al.*,1985). Gynogenesis can also be used extensively in the genetic analysis of crossing over frequencies and gene mapping because the technique of gynogenesis gives precise estimates of cross-over frequencies, allowing construction of good maps. Gynogenesis offers advantages in selective breeding, as gynogenetic offspring are useful for selecting uncommon recessive traits or new mutants. The expression of recessive traits in most populations occurs at a frequency equal to the square of the gene frequency whereas mitotic gynogenetic offspring express a recessive trait with a frequency equal to the gene frequency in the population (Chourrout, 1984).

Artificial gynogenesis is easily induced in fish, but the resulting progeny are usually haploid: diploid progeny result only if the maternal chromosomes are duplicated as described above. These duplicated chromosomes produce a pattern of inheritance that yields offspring of significance to genetics or fish culture. Early work to produce haploid and diploid gynogenesis used x-rays, gamma-rays and uv irradiation in various fish sperm; common carp (Romashov *et al.*, 1960; Nagy *et al.*, 1978), loach (Romashov *et al.*, 1960), sturgeons (Romashov *et al.*, 1963), flatfishes (Purdom, 1969), several salmonids (Purdom, 1969; Lincoln *et al.*, 1974;

Chourrout, 1980; Refstie *et al.*, 1982; Onozato, 1982; Thorgaard *et al.*, 1983; Chourrout, 1984; Thompson and Scott, 1984; Kaastrup and Horlyck, 1987); grass carp (Stanley and Sneed, 1974); medaka (Ijiri, 1980); zebra fish (Streisinger *et al.*, 1981); tilapia (Chourrout and Itskovich, 1983, Penman *et al.*, 1987); paradise- fish (Gervai and Csanyi, 1984); Indian major carps (John *et al.*, 1984); European catfish, *Silurus glanis* (Krasznai and Marian, 1987); *Cirrhinus mirgala* (John *et al.*, 1988) and red sea bream (Sugama *et al.*, 1990). For a review of gynogenesis see section 1.2.4 Chapter 1.

Gynogenetic grass carp (*Ctenopharyngodon idella*) were produced by x-rays or uv-irradiated sperm, or by crossing grass carp with remotely related species. The diploid larvae were produced by gynogenesis, to be used in pratical weed management where assurance against reproduction was needed (Stanley, 1982). In common carp (*Cyprinus carpio* L.), gynogenesis was achieved by cold shocking eggs, fertilized with irradiated sperm, at different times after fertilisation. Consistent yields of 25-50 % viable, gynogenetic fry were obtained when eggs were incubated at 24°C and cold shocked (0°C, 45 min) 1-2 or 7-9 minutes after fertilisation (Komen *et al.*, 1988).

Gynogenesis induction can be coupled with sex inversion to produce XX males (Nagy, 1987; Thorgaard and Allen, 1987). These hormonally sex-reversed gynogenetic males can be useful in crossbreeding experiments to produce all monosex female populations where female fish are more preferable or the growth rate of females is superior to that of males i.e. in cyprinids, clariids and salmonids. Previous research on *Oreochromis niloticus* has shown that a spontaneously sex-reversed XY female and hormone treated genetic males can produceYY male progeny gynogenetically (Scott *et al.*,1989; Mair, 1993).

The main rationale for gynogenesis induction in fish, besides the progressive research in this field has been its potential for rapidly generating inbred lines (Streisinger *et al.*, 1981; Nagy, 1987). The efficiency of meiotic gynogenesis for the production of inbred lines depends on the frequency of recombination between any given gene and its centromere during meiosis. Initially, it was expected that these gene centromere crossing over frequencies would be very low in fish and a very high degree of homozygosity could be produced even in the first generation of meiotic gynogenetics (Nagy, 1987). Therefore, it was believed that meiotic gynogenetic offsprings might be useful for producing inbred lines (Ihssen *et al.*, 1990). In fact, the application of allozyme markers in several studies revealed that the rate of crossing over in the first meiotic metaphase generates high levels of heterozygosity in chromosome regions distant from the centromere (Cherfas, 1977; Streisinger *et al.*, 1981; Thorgaard *et al.*, 1983; Gervai and Csanyi, 1984; Allendorf *et al.*, 1986). The homozygous inbred lines will never be produced by using meiotic gynogenetic diploids, even when such lines have been repeatedly reproduced for several generations (Han *et al.*, 1991). The conventional methods of sib-mating to produce inbred lines required the maintenance of several lines with close inbreeding for up to 20 generations (Purdom and Lincoln, 1973). The suppression of the first cleavage to produce mitotic gynogenetic diploids is considered to be more useful than the meiotic gynogenesis, as it could shorten the time required by producing completely homozygous progeny in the first generation and a fully inbred line in the second generation.

In *Clarias macrocephalus* the application of gynogenesis has been investigated by using UV irradiated sperm of *Pangasius sutchi* (Na-Nakorn *et al.*, 1993). The aims of this research are: to investigate the methods of induction, to optimize the induction factors and to examine the sex of gynogenetic progeny to provide an insight into the sex determinating system in these species.

6.2 Materials and methods

6.2.1 Catfish broodstock

In this study, diploid gynogenesis was induced using eggs from female walking catfish (*C. macrocephalus*) fertililized with African sharp tooth catfish (*C. gariepinus*)sperm that had been genetically inactivated with ultraviolet (UV) light.

6.2.2 Sperm collection

The testes of a *C. gariepinus* male were taken out, minced in a fine nylon net, the milt was transferred to microcentrifuge tube and centrifuged at 1,500 x g at 4°C for 10 min, then the top clear fluid was removed. Approximately 500 µl of undiluted sperm was sampled and kept separately for positive control. A small amount of milt (5-10 µl) was sampled and diluted with Modified Corland' s saline at an appropriate dilution factor depending on initial concentration. The concentrated sperm sample was stored at 4°C.

6.2.3 Sperm motility tests

Generally, spermatozoa are immotile before and after release from the testis. They only become motile once they come into contact with metabolic waste (urine, faeces), mucus and water. If the spermatozoa are motile, then they will die quickly.

The motility of the spermatozoa was checked by mixing 5 µl of milt (diluted from the concentrated sample with Modified Cortland's saline) with 50 µl of water in a microcentrifuge tube and then rapidly placing a drop of this mixture on a glass slide for microscopic examination. Only diluted sperm samples with motility score of 9-10 (on a scale of 0-10) after activation were used.

6.2.4 Estimation of sperm concentration

Sperm concentration was estimated using a Neubauer counter (Haemacytometer, 0.1 mm, 1/400 mm^2, Weber Scientific, England). Before the diluted sperm was used for an experiment, sperm head counts were made to estimate the sperm concentration, 10 µl of the sperm sample was diluted in 490 µl of Modified Cortland's Saline(MCS). From the first dilution, 10 µl was removed and added to a firster 90 µl of MCS making a 1/50 and 1/10 dilution respectively. A small volume of this diluted sperm was placed on the Haemacytometer for counting. Concentrations of 2.5×10^7 ml^{-1} or 2.5×10^8 ml^{-1} were used for irradiation and fertilisation.

6.2.5 Sperm irradiation

Sperm samples were diluted with a deactivator (Modify Cortland's Saline) to give cell concentrations of 2.5×10^7 ml^{-1} or 2.5×10^8 ml^{-1} before irradiation with a 6 watts UV lamp (wave length 254 nm). This was situated approximately 28 cm above the diluted sample fluid which produced a dose rate of 200 μWcm^{-2} (measured by using a UV radiometer, UVP Inc., USA). During irradiation, 2 ml of diluted sperm were placed in a 5.2 cm diameter petri dish which was constantly agitated using an electric stirrer (Jencons Miximatic, USA).

6.2.6 Induced spawning and artificial fertilisation

Sexually mature female *C. macrocephalus* spawn at approximately 6-8 weeks intervals under the experimental conditions described in Chapter 2. Some mature females were implanted with LHRHa to induce development of the eggs as

described in Chapter 2. The readiness of females to spawn was ascertained by examining the degree of swelling of the belly and the colour of the urogenital papilla. Ovalation was induced by injecting the female with LHRHa hormone and domperidone (details described in Chapter 2). After a latency period of 15-18 hours, the eggs were manually stripped immediately after a first batch of eggs had been deposited. Once stripped, the fertilisability with the diluted and UV-light treated milt from macerated testes of the male declined rapidly, probably due to desiccation of the eggs. Since, however, most of the experimental series were time-consuming, it was considered appropriate in some cases that eggs be obtained by sequental stripping over a period of up to 1 hour. Preliminary data suggested that this method of obtaining the eggs resulted in uniformly high fertilisation rates (in agreement with the method used by Nukwan, *et al.*, 1990) . Fertilisation was carried out *in vitro* by mixing 2 ml of diluted sperm (concentration of 2.5 x 10^7 ml^{-1} or 2.5 x 10^8 ml^{-1}) per batch of eggs followed by the addition of 10-20 ml of incubator water (28°C). After collection of eggs female fish were tagged (PIT tag) and kept alive for future experiments.

6.2.7 Experimental design

The effect of UV irradiation on the survival rate of haploid embryos (measured asfertilisation of eggs and survival of embryos at somite and hatch stages) fertilized with UV treated sperm at a concentration of 2.5 x 10^7 ml^{-1} or 2.5 x 10^8 ml^{-1} and subjected to different UV doses (0-32 min) at 200 μWcm^{-2} was carried out through fertilizing the eggs with sperm sample followed by the addition of 10-20 ml water for activation and incubated as described in section 2.3 and 2.5 in Chapter 2.

Control: Three controls were designed; i.e. (i) eggs fertilized with undiluted sperm stock as the primary criterion of success of fertilisation, (ii) eggs fertilized with diluted non-irradiated sperm as a positive control (the hatching rate of normal

larvae is the primary criterion for estimating the relative success of induced gynogenetic diploidization, with all percentages expressed in relation to the initial number of eggs and the hatching rate of the larvae in this control group) and (iii) eggs fertilized with diluted; non- irradiated sperm with cold shocking applied prior to the extrusion of the second polar body (triploid hybrid).

Induction of gynogenetic diploids by suppression of the second meiotic division (early shocking) a fixed cold shock temperature at 2°C, varied time after fertilisation (TAF) from 2-6 min (1 min intervals) and varied shocking duration (D) of 15, 20, 25, or 30 min were tested to attempt to coincide with the second meiotic division and the extrusion of the second polar body in *C. macrocephalus* eggs.

Induction of gynogenetic diploids by suppression of the first cleavage (late shocking): the first cleavage of this hybrid larvae development takes place about 30-35 min after fertilisation under the conditions described in 2.5 Chapter 2, as determined by microscopic observation (See figure 2.8). Cold shock treatments were applied at the approximate time of the first cleavage: a fixed cold shock temperature at 2°C was applied at 1 min intervals from 30-35 min after fertilisation for 20 min duration.

Each experiment was repeated three times (as the replications) using different female brooder fish with the same condition of treated sperm and cold shocking.

6.2.8 Estimation of induction efficiency and control

Fertilized eggs in each treatment and each control (i.e. diploids, diploids positive control and triploids negative control) were incubated separately in small hapas at 28°C in a recirculating water system (as described in section 2.5). The embryos hatched out in 22-26 hours and the survival rate in each group was checked at three

development stages as described in section 2.8. The data were collected and expressed relative to the positive control and about 20 fry in each treatment were sampled for chromosome counting. The fry were reared in the incubation system until three days after hatching, when the survival rate data was collected and expressed in relation to the survival rate of the positive control and about 20 fry derived from each treatment and control were randomly sampled to identify isozyme genotypes of parents and offspring by starch gel electrophoresis. Electrophoresis techniques and allele designation followed procedures and results as described in Chapter 3. A diploid maternal karyotype and the absence of paternal gene expression were the main criteria in determining the success of induced diploid gynogenesis.

6.2.9 Karyological examination

About 20 newly hatched or 1 day-old larvae from each batch were karyotyped by colchicine treatment and counting chromosome, prepared according to the solid-tissue technique of Kligerman and Bloom (1977). Metaphase chromosomes of each individual larvae were counted and recorded. Good metaphase chromosome spreads were photographed through a photo microscope under x 1,000 magnification. For establishing the karyotypes, the best photographs were used for cutting out, pairing and classifying chromosomes in increasing size (details described in section 2.10.1).

6.2.10 Electrophoretic confirmation

About 20 fry (three days post-hatch) from each batch were sampled, obtained alive and killed by refrigerating for 30 minutes, homogenized with homogenizing buffer, frozen and stored at -20°C until tested. Electrophoresis was carried out and followed the methods used by Sodsuk and McAndrew (1991), details as described

in section 2.11. The identification of the different alleles at the same locus was decided by the banding position of isozymes in the same gel.

6.2.11 Sexing and gonad development

Viable 3 day old gynogenetic and control fry were transferred from the incubating system into 25 *l* plastic aquaria. During that time the fry were fed with *Artemia* nauplii. At 7 days after hatch, gynogenetic (pooled between treatments), diploid hybrid, triploid hybrid and pure *C. macrocephalus* fry were each stocked into 40 *l* round plastic tanks in a controlled system and left for on-growing at a density of less than 100 fry/tank for 3 months (section 2.3.1). The early fry and on-growing fish were fed three and two times daily respectively with the recommended amount of various sizes of trout feeds (section 2.3.2). The experiment was terminated at 3 months or more, when all the fish belonging to the four treatment groups were manually sexed on the basis of the morphology of the urogenital papilla to determine the sex ratio (section 2.12). All the fish were then killed and where possible, fish were also sexed by examining the gonads. Unidentified gonads were transferred to Bouin's fluid overnight before being finally fixed in 70 % ethanol. Dehydrating, paraffin embedding and sectioning and staining of gonadal samples were made according to the recommended procedures (Appendix 6). General histological examination was carried out under x100, x250, x400 and x1,000 (oil immersion) magnifications and photographed through an Orthomat Photomicroscope. It was necessary in this study to check the sexes of all viable gynogenetic diploids, including immmature young fish, derived from all the experimental trials along with controls in an attempt to determine their sexual status. The estimation of growth body weight and standard length were made on a sampling from each treatment group. All the sexed data are given as percentages of each sex observed from each treatment group.

6.3 Results

6.3.1 Determination of the effects of UV irradiation on the motility of the sperm

Sperm motility scores at two different concentrations, $2.5 \times 10^7 ml^{-1}$ and $2.5 \times 10^8 ml^{-1}$, subjected to various durations of a fixed 254 nm UV irradiation at 200 μWcm^{-2} are shown in Figure 6.1. This was done in 3 replicates. For concentration of $2.5 \times 10^7 ml^{-1}$ (Figure 6.1a), a marked decline in motility (lower than 50 %) was observed when it had been exposed to UV light for ≥2 min. A zero motility score was observed after it had been irradiated for 16 min. At concentration $2.5 \times 10^8 ml^{-1}$ (Figure 6.1b), motility started to decline to lower than 50 % when the spermatozoa had been exposed to UV light for 4 min. Subsequently, zero motility was observed after it had been irradiated for 32 min. These results showed that the duration of UV irradiation and sperm concentration affected the motility of spermatozoa.

6.3.2 Determination of UV irradiation on survival of haploid embryo

Mean fertilisation and survival (relative to control) of embryos at somite and hatch stages from eggs fertilized with two concentrations of sperm treated with UV for different durations are given in Table 6.1-6.2 and Figure 6.2. At sperm concentrations of $2.5 \times 10^7 ml^{-1}$ and $2.5 \times 10^8 ml^{-1}$, a decline in the number of somite stage embryos fertilized with UV treated sperm relative to the control was observed at the lowest UV dose, 0.25 min. As the UV irradiation dosage was increased embryo survival increased and then declined to zero at the maximum dose of 32 min. The dose yielding the highest frequency of hatched embryos for concentration $2.5 \times 10^7 ml^{-1}$ was 1 min and for concentration $2.5 \times 10^8 ml^{-1}$ was 2 min No viable haploid larvae survived after hatch out for more than a few hours.

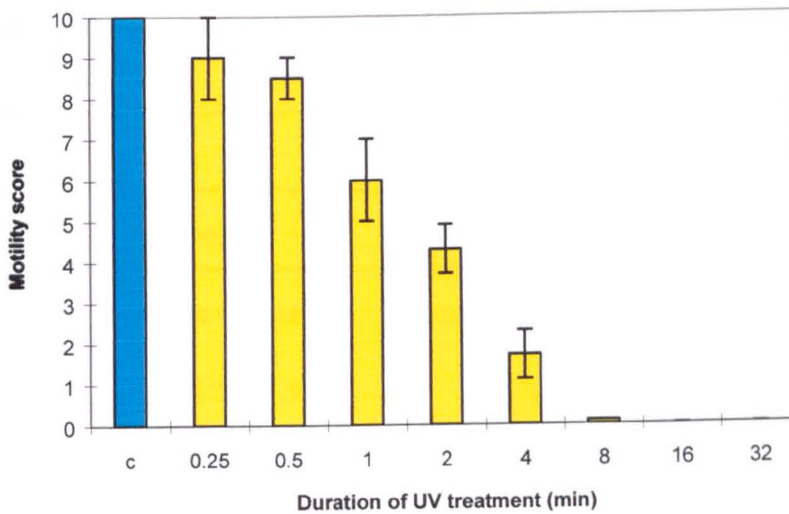
Based on these results (the embryo survival to hatch out appeared to decline at lower UV dose, increased as the UV irradiation exposure was increased and then continuously declined to zero at the maximum dose) a pseudo-Hertwig effect was observed at both concentrations. Mean fertilisation and survival at somite and hatch stage of the control (untreated sperm) with the two sperm concentrations are shown in Table 6.1 and Table 6.2.

6.3.3 Determination of optimum time after fertilisation and duration for cold shocks.

The variation of fertilisation rate, survival rate to somite stage of larval development, survival rate to hatch out and survival rate to 3 days of age for each treatments are shown in table 6.3 and figure 6.3-6.6. These results were derived from 3 repeated experiments using three different females.

The *C. macrocephalus* eggs fertilized with normal sperm of *C gariepinus* (diploid hybrid) show normal viability and the hatching rate was comparable to that of normal hybrid production: the survival rate of the triploid hybrid control was very low (as seen for this treatment in the results in chapter 5). The average fertilisation rate and hatching rate of the diploid controls were 88.03 ±6.34 % and 44.62 ±16.40 % respectively, indicating good quality of eggs and sperm, as for normal artificial insemination. Eggs fertilized with UV-irradiated sperm of the African catfish resulted in hatching of only abnormal larvae (usually eye not developed, mouth closed and head deformed), with these abnormalities known generally as the haploid syndrome. The non-viability of these individuals indicates that the paternal DNA in the UV-irraiated sperm were indeed genetically inactivated (Chourrout, 1982).

(A)



(B)

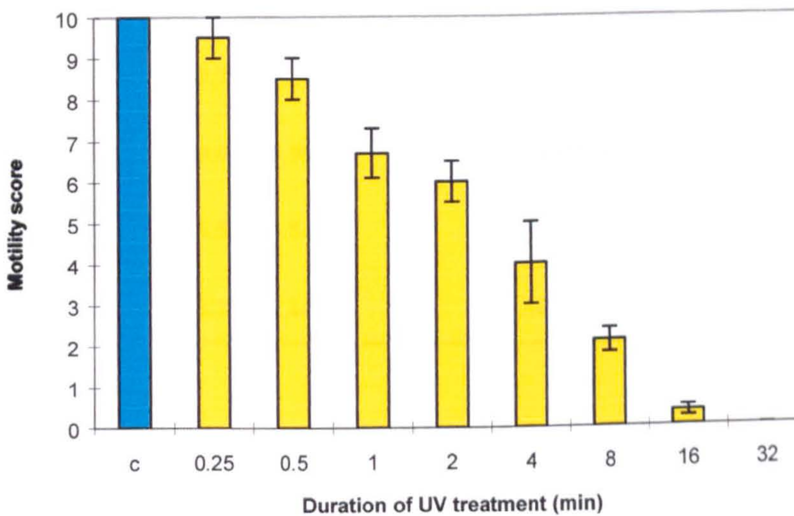


Figure 6.1 Sperm motility scoring subjected to different UV doses (min) at sperm concentration (A) 2.5 x 107 cell ml-1 and (B) 2.5 x 108 cell ml-1. A motility score of 10 denoted that 100% of spermatozoa under observation were motile and moving actively, while a 0 score indicated that no sperm were moving after activation. Controls received no UV treatment.

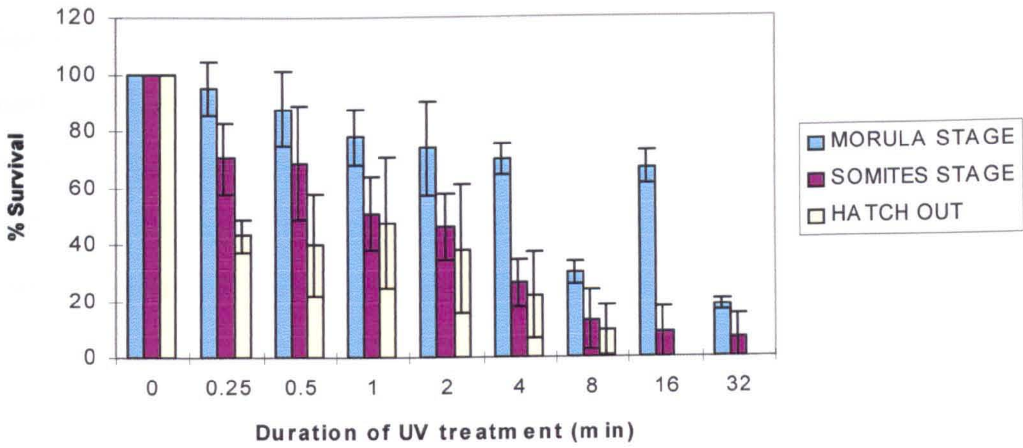
Table 6.1 Mean survival to morula, somite and hatch stage of haploid embryos fertilized with UV treated sperm at concentration of $2.5x10^7$ ml^{-1}

UVdose	%RC±SE (%Control±SE)		
(min)	Morula	Somite stage	Hatch out
0	100(64.98±3.52)	100(63.73±10.11)	100(49.02±2.58)
0.25	94.78±09.56	70.32±12.44	43.16±05.68
0.5	81.75±13.40	68.67±20.06	39.73±18.09
1	77.67±06.93	50.90±12.61	47.53±22.79
2	73.61±16.50	45.87±11.61	38.20±22.83
4	69.72±05.54	17.05±07.27	21.91±15.17
8	30.09±03.99	13.11±10.40	9.54±08.84
16	16.53±05.84	8.53±08.88	0
32	18.17±02.16	6.54±08.16	0

Table 6.2 Mean survival to morula, somite and hatch stage of haploid embryos fertilized with UV treated sperm at concentration of 2.5x 10^8 ml^{-1}

UVdose	%RC±SE (%±SE)		
(min)	Morula	Somite stage	Hatch out
0	100(79.66±4.84)	100(62.47±14.83)	100(49.62±17.76)
0.25	91.22±6.41	52.48±13.49	19.62±12.57
0.5	94.93±8.17	50.75±15.72	14.16±11.45
1	83.14±7.00	62.32±6.78	9.38±11.89
2	78.53±10.53	64.81±13.45	15.02±11.32
4	73.28±7.76	50.33±18.62	1.02±0.84
8	41.75±14.74	24.50±20.63	0
16	23.38±6.36	4.38±3.41	0
32	11.32±6.64	2.88±4.99	0

(A)



(B)

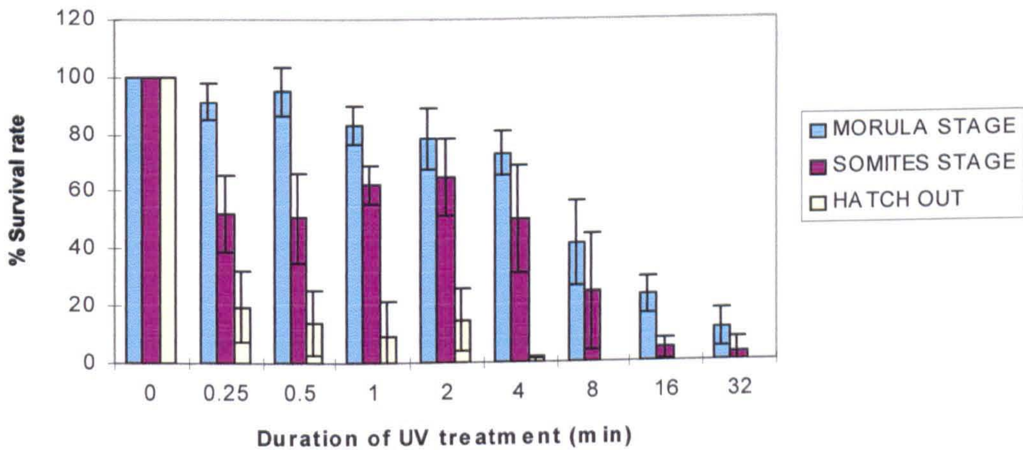


Figure 6.2 Survival of haploid embryos at different development stages (means of 4 replicates) UV (254 nm) dose treated at 200 μW cm^{-2}. Concentration of sperm (A) 2.5 x 10^{7} cell ml^{-1} (B) 2.5 x 10^{8} cell ml^{-1}. All results are expressed relative to the non-irradiated control.

In cold shock treated eggs, the fertilisation rate and hatching rate of normal larvae varied with the treatments. A fixed cold shock at 2° C for longer than 30 min caused 100 % mortality in larvae at 3 days of age (yolk sac resorption). The highest fertilisation rate relative to the control after adjustment of the latter to 100% (85.30 ±1.42%) and the highest survival to 3 days of age (28.57±9.49%) were obtained in the shock treatment 2° C for 15 min applied 4 min after fertilisation (Table 6.3).

6.3.4 Induction of diploid mitotic gynogenesis

The fertilisation rate and hatching rate of larvae were very poor in treatments applied at the approximate time of the first cleavage (Table 6.4). A hatching rate of more than 2 % was found in the treatment applied at 2° C for 20 min begining 35 min after fertilisation, but all hatched larvae were deformed and died several hours after hatching. Several combinations of time after fertilisations and durations were tested to attempt to suppress the first cleavage, but the survival rate of larvae was still very poor.

6.3.5 Efficiency of gynogenetic diploid induction

Electrophoretic analysis of parental species of *C. gariepinus* and *C. macrocephalus* confirmed that there are at least 3 fixed differences at enzyme loci between them i.e., *GPI-2**, *LDH-1** and *MDH-2** loci. Allelic contributions from both parental species were observed in every diploid hybrid (20 individuals each batch of experiments) at each of the loci showing parental allelic differences, confirming that these fish were not spontaneous androgenetic or gynogenetic offsprings (Figure 6.7). However, paternal alleles did not appear in

Table 6.3. The effect of different times after fertilisation (TAF) and durations (D) of application of fixed cold shock at 2°C on gynogenetic diploid induction in walking catfish (*C. macrocephalus*) using UV treated sperm of the African catfish (*C. gariepinus*).

Treatments				Expected larvae	Fertilisation rate %RC#±SE	Somite stage %RC#±SE	Hatch out %RC#±SE	3 days of age %RC#±SE
UV treated sperm	Cold shock at 2°C	TAF (min)	Duration (min)					
-	-	-	-	2n	100^{a}	100^{a}	100^{a}	100^{a}
-	+	2	30	3n	58.99±10.80^{b}	14.41±5.81^{b}	5.49±4.87^{c}	3.29±2.70^{c}
+	+	2	15	2n*	84.18±5.29^{ab}	32.33±15.96^{ab}	2.46±1.48^{c}	2.93±1.01^{c}
+	+	2	20	2n*	84.89±6.97^{ab}	38.48±17.59^{ab}	2.06±1.61^{c}	1.51±1.12^{c}
+	+	2	25	2n*	74.31±9.57^{ab}	24.89±13.86^{b}	0^{c}	0^{c}
+	+	2	30	2n*	64.07±6.22^{ab}	24.19±9.28^{b}	0^{c}	0^{c}
-	+	3	30	3n	77.33±10.59^{ab}	22.05±13.19^{b}	2.18±1.24^{c}	1.51±0.76^{c}
+	+	3	15	2n*	71.55±3.94^{ab}	32.38±14.64^{ab}	6.03±4.01^{c}	5.97±3.78^{c}
+	+	3	20	2n*	74.23±0.79^{ab}	32.78±19.09^{ab}	5.17±3.69^{c}	4.46±3.13^{c}
+	+	3	25	2n*	78.33±6.99^{ab}	25.23±20.35^{b}	0.32±0.32^{c}	0.27±0.27^{c}
+	+	3	30	2n*	74.35±8.26^{ab}	28.59±21.42^{ab}	0.64±0.64^{c}	0.55±0.55^{c}
-	+	4	30	3n	81.07±4.69^{ab}	43.99±16.88^{ab}	13.83±4.04^{ab}	10.87±2.49^{c}
+	+	4	15	2n*	85.30±1.41^{ab}	46.39±13.44^{ab}	41.23±21.16^{b}	28.57±9.49^{b}

Table 6.3. (cont.)

Treatments				Expected larvae	Fertilisation rate %RC#±SE	Somite Stage %RC#±SE	Hatch out %RC#±SE	3 days of age %RC#±SE
UV treated sperm	Cold shock at 2°C	TAF (min)	Duration (min)					
+	+	4	20	2n*	81.73±5.80^{ab}	43.19±15.53^{ab}	22.45±15.92^{ab}	15.64±9.88^{bc}
+	+	4	25	2n*	71.87±7.20^{ab}	28.38±12.02^{ab}	6.00±3.72^{c}	4.53±2.50^{c}
+	+	4	30	2n*	69.20±3.51^{ab}	16.98±6.40^{b}	0.42±0.42^{c}	0^{c}
-	+	5	30	3n	72.55±1.27^{ab}	42.60±16.88^{ab}	5.93±0.26^{c}	5.26±0.40^{c}
+	+	5	15	2n*	78.96±3.81^{ab}	40.57±14.05^{ab}	11.13±0.84^{ab}	11.32±0.31^{c}
+	+	5	20	2n*	78.01±1.88^{ab}	32.57±14.17^{ab}	9.81±0.80^{c}	8.44±0.20^{c}
+	+	5	25	2n*	71.78±6.40^{ab}	29.44±11.59^{ab}	7.35±1.28^{c}	4.55±1.23^{c}
+	+	5	30	2n*	60.92±7.54^{b}	10.65±2.59^{b}	0^{c}	0^{c}
-	+	6	30	3n	73.78±0.90^{ab}	22.41±8.08^{b}	3.34±1.73^{c}	2.73±1.37^{c}
+	+	6	15	2n*	72.25±1.83^{ab}	19.36±7.01^{b}	5.85±1.89^{c}	4.64±2.33^{c}
+	+	6	20	2n*	61.64±1.82^{b}	14.76±7.15^{b}	2.97±1.51^{c}	2.49±1.24^{c}
+	+	6	25	2n*	73.21±4.72^{ab}	15.63±6.70^{b}	0.42±0.42^{c}	0^{c}
+	+	6	30	2n*	62.87±4.82^{b}	16.99±3.28^{b}	0^{c}	0^{c}

Superscripts denote a significant difference by ANOVA at $p \leq 0.05$ between values in each development stage. 2n*=Gynogenetic diploid hybrid;

RC# Relative to control after adjustment of the latter to 100% (mean survival rate to hatch out in controls was 44.62±16.44).

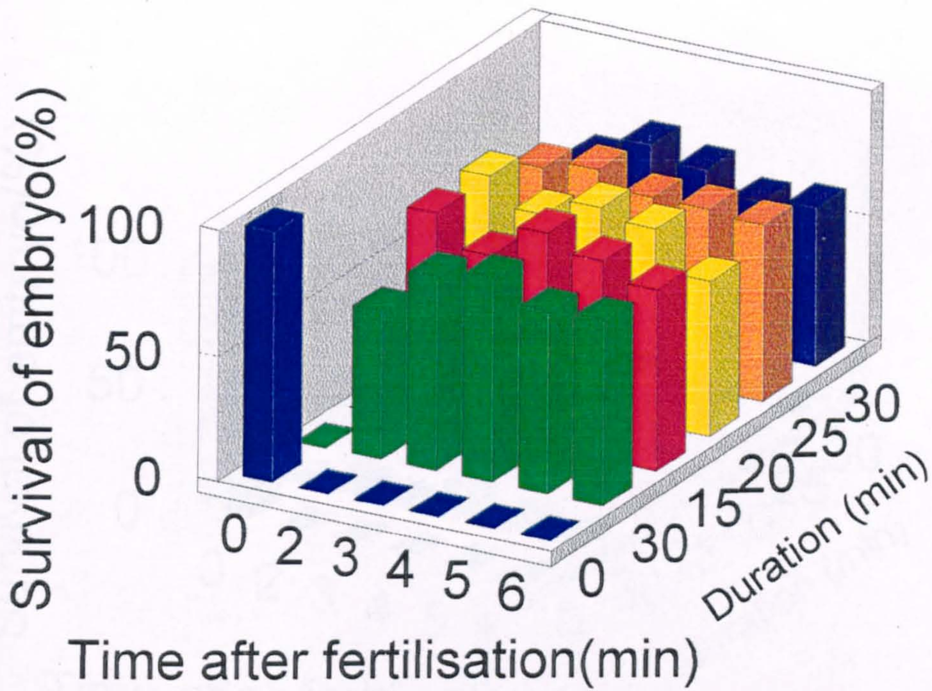


Figure 6.3 The survival rate (%relative to control) at morula stage of *C.macrocephalus* eggs fertilized with UV treated sperm of *C.gariepinus* and a fixed cold shock at 2°C applied at various times after fertilisation and durations

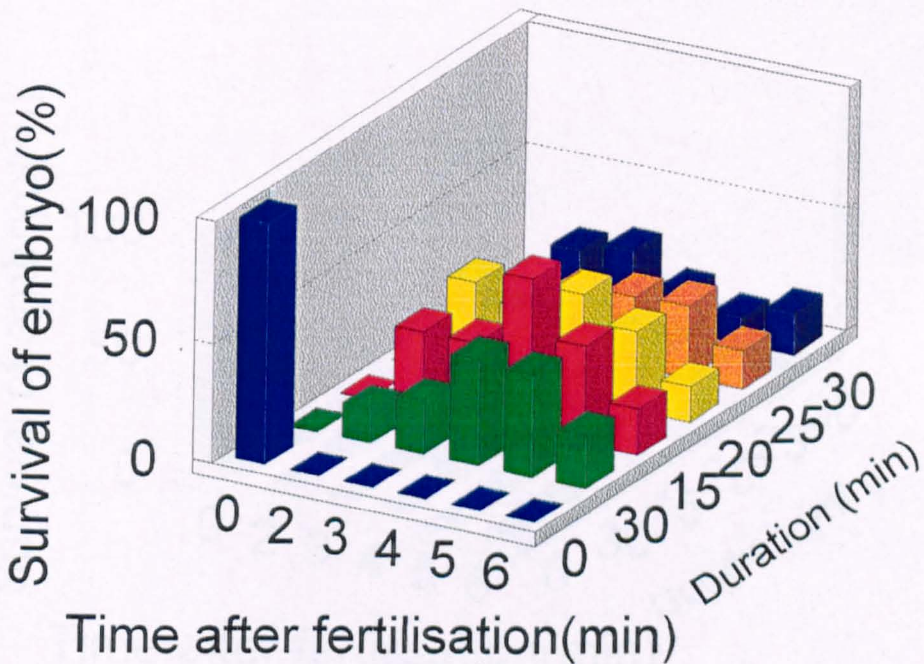


Figure 6.4 The survival rate (%relative to control) at somite stage of *C.macrocephalus* eggs fertilized with UV treated sperm of *C.gariepinus* and a fixed cold shock at 2°C applied at various times after fertilisation and durations

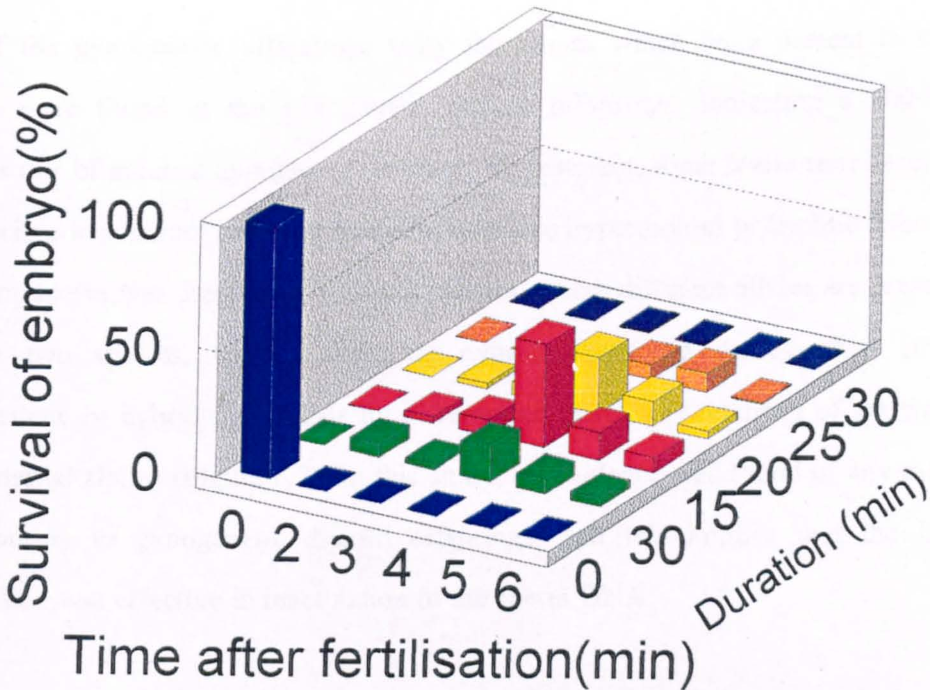


Figure 6.5 The survival rate (%relative to control) at hatching stage of *C.macrocephalus* eggs fertilized with UV treated sperm of *C.gariepinus* and a fixed cold shock at 2°C applied at various times after fertilisation and durations

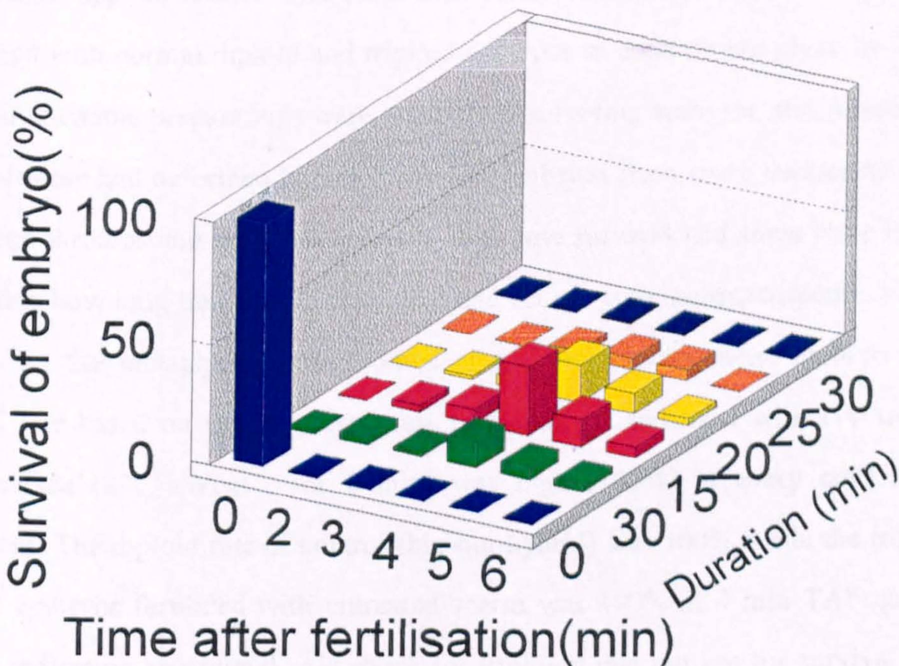


Figure 6.6 The survival rate (%relative to control) at 3 days old of gynogenetic diploids produced from *C.macrocephalus* eggs fertilized with UV treated sperm of *C.gariepinus* and a fixed cold shock at 2°C applied at various times after fertilisation and durations

any of the gynogenetic offsprings. Only the alleles which were present in the female were found in the gynogenetic diploid offsprings, indicating a 100 % success rate of induced gynogeneis. During UV treatment, some sperm may receive insufficient irradiation, and may caused to produce hyperdiploid or triploid hybrid. If donor sperm was used from different species, where different alleles are present in the two species, triploid offspring hybrids could be distinguished from gynogenetic or hybrid individuals by differencies in relative intensity of maternal and paternal alleles (Figure 6.7). In this study, no evidence was found of any male contribution in gynogenetic diploid offsprings, and this implies that the UV irradiation was effective in inactivation of the sperm DNA.

Karyotypes of the embryos from gynogenetic induction experiments with control diploid big-oui hybrids and triploid hybrids were examined from 3 replications of each experiment. The karyotype scores of gynogenetic embryos from cold shocking experiments applied at difference times after fertilisation and durations of shocking compared with normal diploid and triploid embryos as controls are given in Table 6.5. Chromosome preparations were made from surviving embryos after hatch out. Most of these had deformed bodies. Very few embryos from some treatments were taken for chromosome preparation due to their low survival and some were left to determine how long they would stay alive and for on-growing experiments. Figure 6.8 shows the metaphase chromosomes of a diploid gynogenetic embryo. The diploid rate based on the karyotype result of embryos fertilized with UV treated spermatozoa (200 μWcm^{-2}, for 2 min) was high (100%) at every cold shock treatment. The diploid rate of control (big-oui hybrid) was 100% while the triploid rate of embryos fertilized with untreated sperm was 100% at 4 min TAF and 30 min D indicating an optimal cold shock for triploidy rate but not for survival rate.

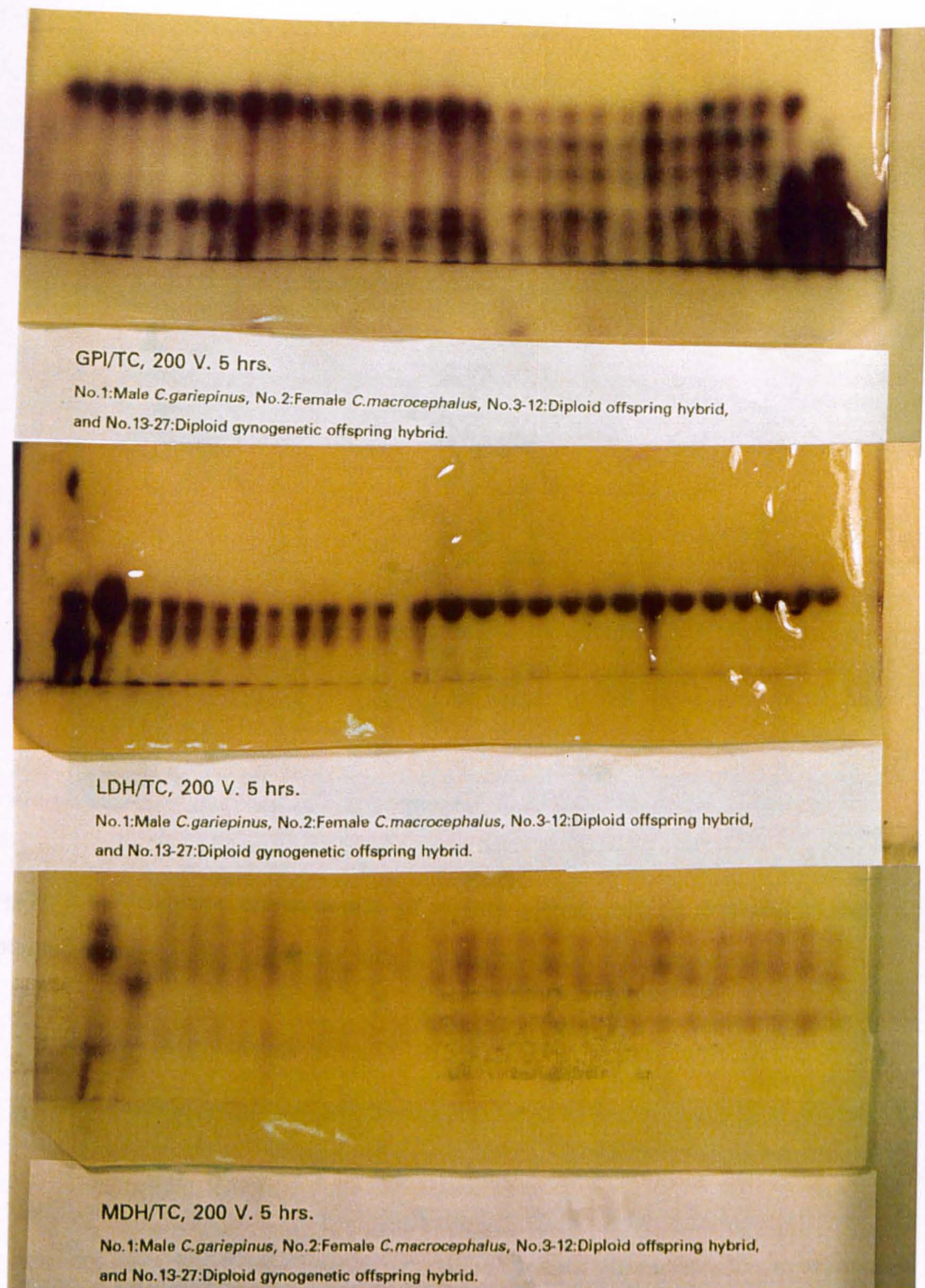


Figure 6.7 Zymograms of *GPI-2**, *LDH-1** and *MDH-2**, illustrating the banding patterns of the African catfish, walking catfish, diploid big-oui hybrid and gynogenetic diploid walking catfish.

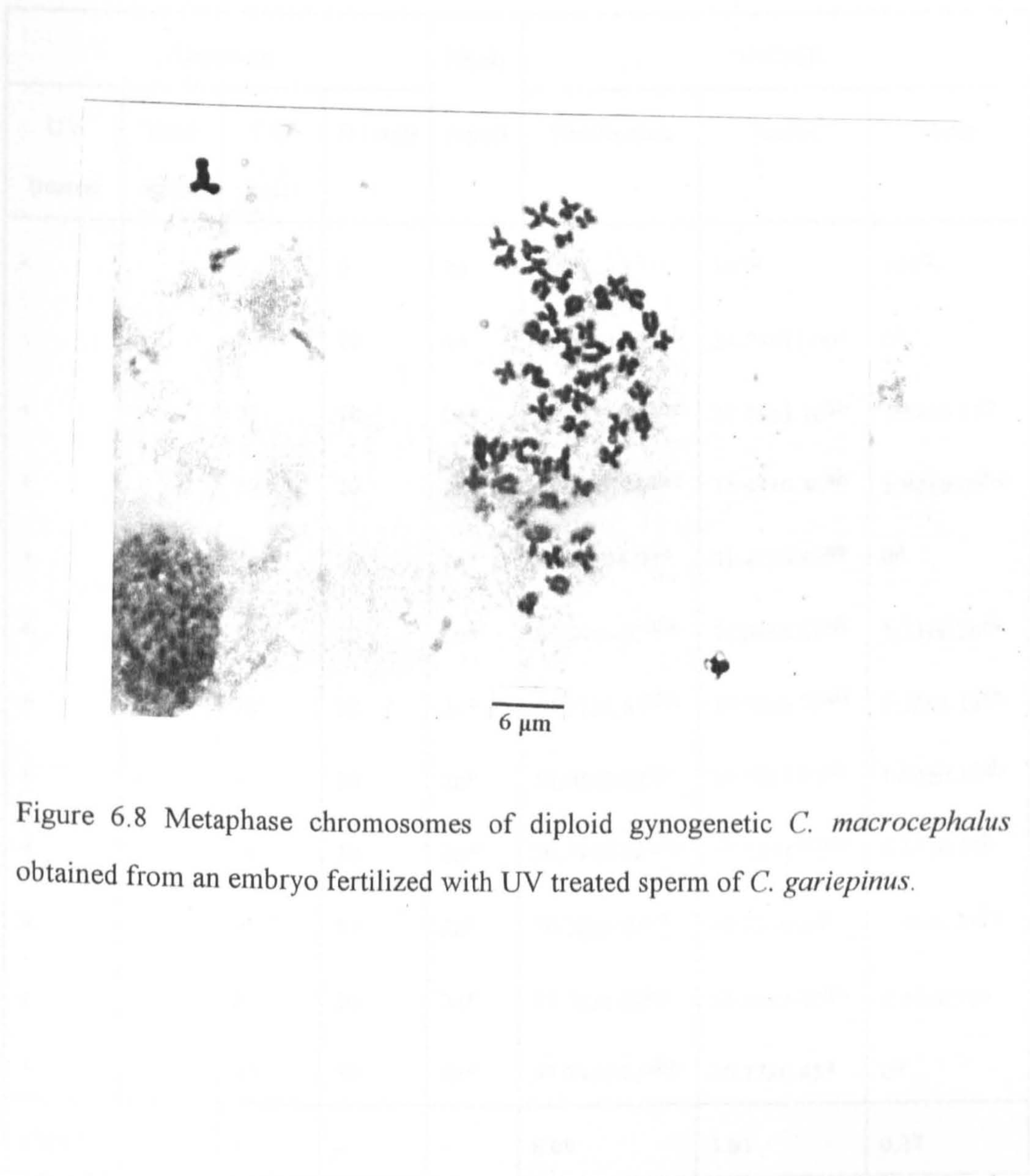


Figure 6.8 Metaphase chromosomes of diploid gynogenetic *C. macrocephalus* obtained from an embryo fertilized with UV treated sperm of *C. gariepinus*.

Table 6.4 Mean fertilisation and survival rate (relative to control) at different development stages of 2n gynogenetic larvae from late shocking experiment (data from 2 replications)

Treatment				Ploidy	%RC±SE		
UV treated	Cold shock	TAF (min)	D (min)	expect	Fertilisation	Somite	Hatch
-	-	0	0	2n	100^{d}	100^{c}	100^{d}
-	+	30	20	4n	49.69 ± 16.23^{ab}	24.54 ± 11.06^{a}	0^{a}
+	+	30	10	2n*	63.06 ± 6.98^{abc}	37.76 ± 1.16^{ab}	1.54 ± 0.33^{b}
+	+	30	20	2n*	64.62 ± 8.46^{abc}	35.43 ± 0.86^{ab}	1.92 ± 0.09^{bc}
+	+	30	30	2n*	44.87 ± 14.00^{a}	31.41 ± 4.83^{ab}	0^{a}
+	+	31	20	2n*	60.94 ± 6.03^{abc}	31.89 ± 9.17^{ab}	1.13 ± 0.26^{ab}
+	+	32	20	2n*	64.21 ± 4.83^{abc}	35.92 ± 6.75^{ab}	1.12 ± 1.12^{ab}
+	+	33	20	2n*	51.08 ± 2.02^{ab}	38.36 ± 7.03^{ab}	1.09 ± 0.17^{ab}
+	+	34	20	2n*	59.71 ± 7.56^{abc}	37.12 ± 4.97^{ab}	1.31 ± 0.30^{b}
+	+	35	10	2n*	76.28 ± 3.24^{cd}	44.21 ± 0.24^{b}	1.95 ± 0.29^{bc}
+	+	35	20	2n*	73.52 ± 4.98^{bc}	35.50 ± 7.47^{ab}	2.71 ± 0.18^{c}
+	+	35	30	2n*	59.20 ± 7.21^{abc}	25.77 ± 0.45^{a}	0^{a}
±SEM	-	-	-	-	8.09	5.83	0.37

2n* gynogenetic diploid catfish

Superscripts denote a significant difference by ANOVA at $p<0.05$ between values in each development stage

Table 6.5 Karyotype scores of the embryos fertilized with untreated and UV irradiated spermatozoa with concentration $2.5x10^8$ cell.ml^{-1} followed by cold shock applied at 2° C with various TAF and durations (data from 3 replications).

UVdose	Cold shock at 2°C			No. of	Expect	Karyotype	
(min)	shock	TAF(min)	D (min)	larvae	ploidy	%2n	%3n
0	-	0	0	60	2n	100	0
0	+	2	30	13	3n	15.38	84.62
2	+	2	15	18	2n*	100	0
2	+	2	20	5	2n*	100	0
2	+	2	25	0	2n*	0	0
2	+	2	30	0	2n*	0	0
0	+	3	30	11	3n	18.18	81.82
2	+	3	15	60	2n*	100	0
2	+	3	20	52	2n*	100	0
2	+	3	25	8	2n*	100	0
2	+	3	30	4	2n*	100	0
0	+	4	30	47	3n	0	100
2	+	4	15	60	2n*	100	0
2	+	4	20	60	2n*	100	0
2	+	4	25	16	2n*	100	0
2	+	4	30	3	2n*	100	0
0	+	5	30	22	3n	0	100
2	+	5	15	60	2n*	100	0
2	+	5	20	35	2n*	100	0
2	+	5	25	14	2n*	100	0
2	+	5	30	0	2n*	0	0
0	+	6	30	27	3n	0	100
2	+	6	15	32	2n*	100	0
2	+	6	20	8	2n*	100	0
2	+	6	25	2	2n*	100	0
2	+	6	30	0	2n*	0	0

2n* gynogenetic diploid

6.3.6 Effects of gynogenetic induction

The data for mean survival rate of gynogenetic embryos at hatch stage and 3 days of age are presented in Table 6.3. Very few embryos were left alive at yolk sac resorption while most surviving embryos could not swim up due to their body deformation. Figure 6.9 shows the features of abnormality of diploid gynogenetic embyos. These abnormalities might came from inbreeding or the effect of UV irradiation or cold shock. However, from the results shown in section 6.3.2, it can be concluded that the UV irradiation had efficiently eliminated the paternal contribution in all doses over 2 min of irradiation.

The effect of UV treatments on the survival to 3 day old embryos were found significant difference with cold shock at 2°C, 4 min after insemination for 15-30 min duration while control triploid embryos have significant lower survival rate than the control big-oui hybrid (see Table 6.3).

Experiments in the previous chapter (Chapter 5) revealed that cold shock was the best agent to induce triploidy. Following on from this, a more comprehensive study of cold shock treatments was made to find the optimum dose for gynogenetic induction. Following the work used a fixed temperature at 2°C, four durations (15, 20, 25 and 30 min) and various times afterfertilisation (2-6 min). These shock parameters gave high results in the present trials. Viable triploid hybrids could be induced and are therefore important controls to show the lethality and deleterious effect of cold shock on embryonic survival at this particular shock intensity and durations. This study revealed that cold shock at 2°C induces 100% triploidy in control but long shocking duration increases the number of deformed embryos and results in lower survival in diploid gynogenetic offsprings. About 10-25 % of embryos in each batch of gynogenetic diploids had deformed bodies. Most of these could not swim up and eat food after yolk sac resorption and died later. Several

batches of normal gynogenetic diploids were reared until at least 3 months of age for examination of sexes and gonadal development compared with control trials. The gynogenetic diploid catfish had the morphological characteristics of pure *C. macrocepalus* but about 5-10 % in every batch of fish were abnormal and blind (Figure 6.10).

6.3.7 Sex ratio and gonadal condition

The sex ratio of pooled experimental fish of four groups (diploid big-oui hybrid, triploid big-oui hybrid, gynogenetic diploid *C. macrocephalus* and control *C.macrocephalus*) was determined at the end of each experiment (maximum age of fish 135 days). The observed frequency of male and female sexes in triploid big-oui hybrid and gynogenetic diploid *C. macrocephalus* were significantly different from the expected 1:1 ratio while diploid big-oui hybrid and pure *C. macrocephalus* were not significantly different from this ratio (Table 6.6). The sex ratios of these four groups of fish are shown in Figure 6.11.

The final observations of morphological difference and gonadal condition in grow out fish in each groups were made at the end of the experiment in 9 batches of gynogenetic diploid fish. The diploid gynogenetic offspring were morphologically the same as the control *C. macrocephalus*, most obviously at the occipital process (Figure 6.12a). The comparision of gonadal condition in diploid big-oui hybrid, dipolid gynogenetic offspring, control *C. macrocephalus* and pure *C. gariepinus* at 135 days of age is shown in Figure 6.12b. In diploid big-oui hybrid, female ovaries were mostly thin, string-like and contained mainly undeveloped oogonia and oocytes, while diploid gynogenetic fish were all female with larger of ovaries full of developing eggs. Both control *C. macrocephalus* and pure *C. gariepinus* had developed gonads in both sexes. In 2n diploid big-oui, male testes were thread-like with undeveloped spermatozoa.

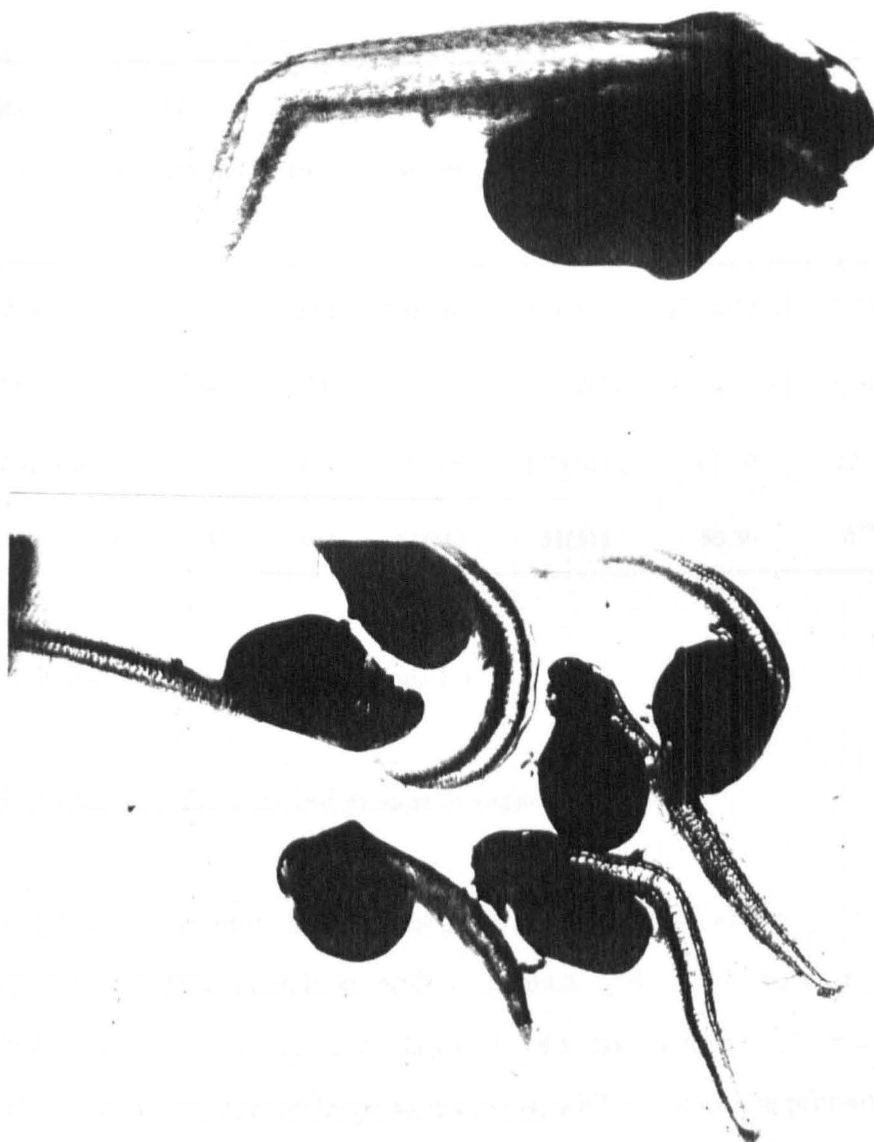


Figure 6.9 Body deformation of embryos produced by diploid gynogenetic induction (70x magnification).

Table 6.6 Observed frequency of male and female sexes and sex ratio of four groups of 9 experimental batches at 3-4½ months of age. Numbers in parentheses are expected value of both sexes with a null hypothesis of a 1:1 sex ratio.

Species	No.of batches	No. fish	No.male (expect)	No.female (expect)	% sex ratio male:femal e	Chi-square (sig. level)
2n hybrid	9	212	101(106)	111(106)	47.64:52.36	3.84* ($p<0.05$)
3n hybrid	9	55	13(22.5)	42(22.5)	23.64:76.36	20.91*($p<0.05$)
2n gynogenetic	9	153	0(76.5)	153(76.5)	0:100	153* ($p<0.05$)
C.macrocephalus	4	102	51(51)	51(51)	50:50	0^{NS} ($p>0.05$)

* $p<0.05$

N.S. = Not significantly different from 1:1

6.3.8 Gonadal histology of four groups of experimental fish.

Histological sections of diploid big-oui and triploid hybrid's ovaries at 3-4½ months of age showed they contained mainly oogonia and a few small primary or previtellogenic oocytes (Figure 6.13a-b). At this age, most of the cells were of similar size with very few undergoing divisions, while some of the primary oocytes had started to show retarded development. Histological sections of diploid gynogenetic ovaries at 3-4½ months of age had a strongly basophillic cytoplasm and lightly stained round nuclei in developing primary and previtellogenic oocytes (Figure 6.13c).

Histological sections of both diploid big-oui and triploid hybrid testes contained very few cysts with spermatogonia and spermatocytes. Most testicular sections of

triploid hybrids had very rare germ cells which under active divisions and had developed into spermatozoa. The testicular sections of pure *C. macrocephalus* contained highly distinct cysts surrounded by the basal lamina at all stages of development. There were spermatogonia, primary spermatocytes and a few spermatozoa in this age of fish (Figure 6.14).

6.4 Discussion

Artificial induction of diploid gynogenesis has been reported in many fishes (Purdom, 1983; Thorgaard *et al.*, 1983; Ihssen *et al.*, 1990). The optimum conditions for producing the diploid gynogenetics of *C. macrocephalus* by cold shock, using the UV treated sperm of *C. gariepinus,* were demonstrated in this study. The African sharptooth catfish, *C. gariepinus* was employed as a donor fish for foreign sperm in this study because usually *C. gariepinus* was used as male brooder in big-oui hybrid production and because the use of foreign sperm for induced gynogenesis has advantages in the case of confirming that paternal genes are not transmitted. It is essential in gynogenesis research to use sperm from male fish which has distinguishable alleles from the female at at least one locus, allowing the presence of any male contribution to be detected by electrophoresis (Thorgaard, *et al.*, 1983). For this reason, normal fry achieved from eggs fertilized with UV irradiated sperm were identifiable as gynogenetic diploids of *C. macrocephalus*. Similar methods were reported by Suzuki, *et al.*, (1985); Hollebecq, *et al.*, (1986); Sugama, *et al.*, (1990); Taniguchi, et al. (1991); Fujioka (1993) and Pongthana, *et al.*, (1995).

In this study, the Hertwig effect was clearly demonstrated. A UV dose of 200 μWcm^{-2} for 2 min seems to be adequate to genetically inactivate *C. gariepinus* sperm at the sperm concentrations used.

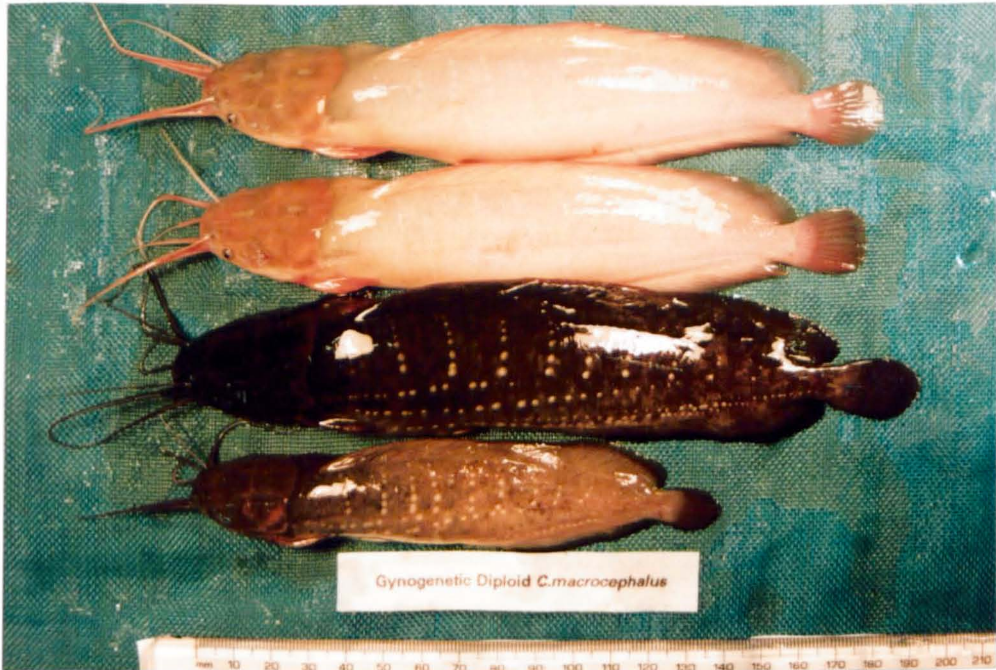


Figure 6.10 Normal gynogenetic diploid *C. macrocephalus* at 4 months old (upper two fish); the dark coloured fish (lower two fish) are abnormal and blind.

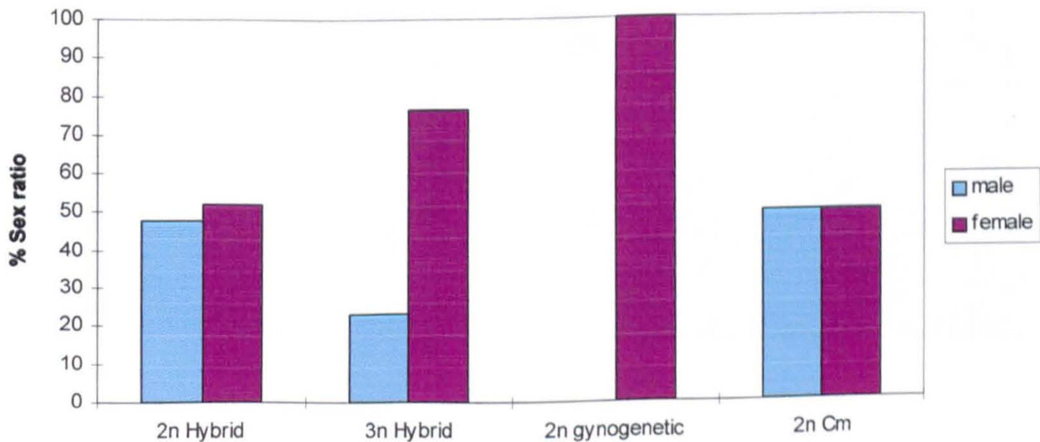


Figure 6.11 Sex ratios in diploid hybrid, triploid hybrid, diploid gynogenetic catfish and diploid *C. macrocephalus*.

Figure 6.12 Morphological differences and gonadal condition in gynogenetic diploid *C. macrocephalus*, diploid big-oui hybrid, pure *C. macrocephalus* and pure *C. gariepinus* (A): occipital process (white arrow pointed) and white round transverse bands along bodyside (sharp arrow pointed) (B): gonadal condition showing testis (T) and ovary (O).

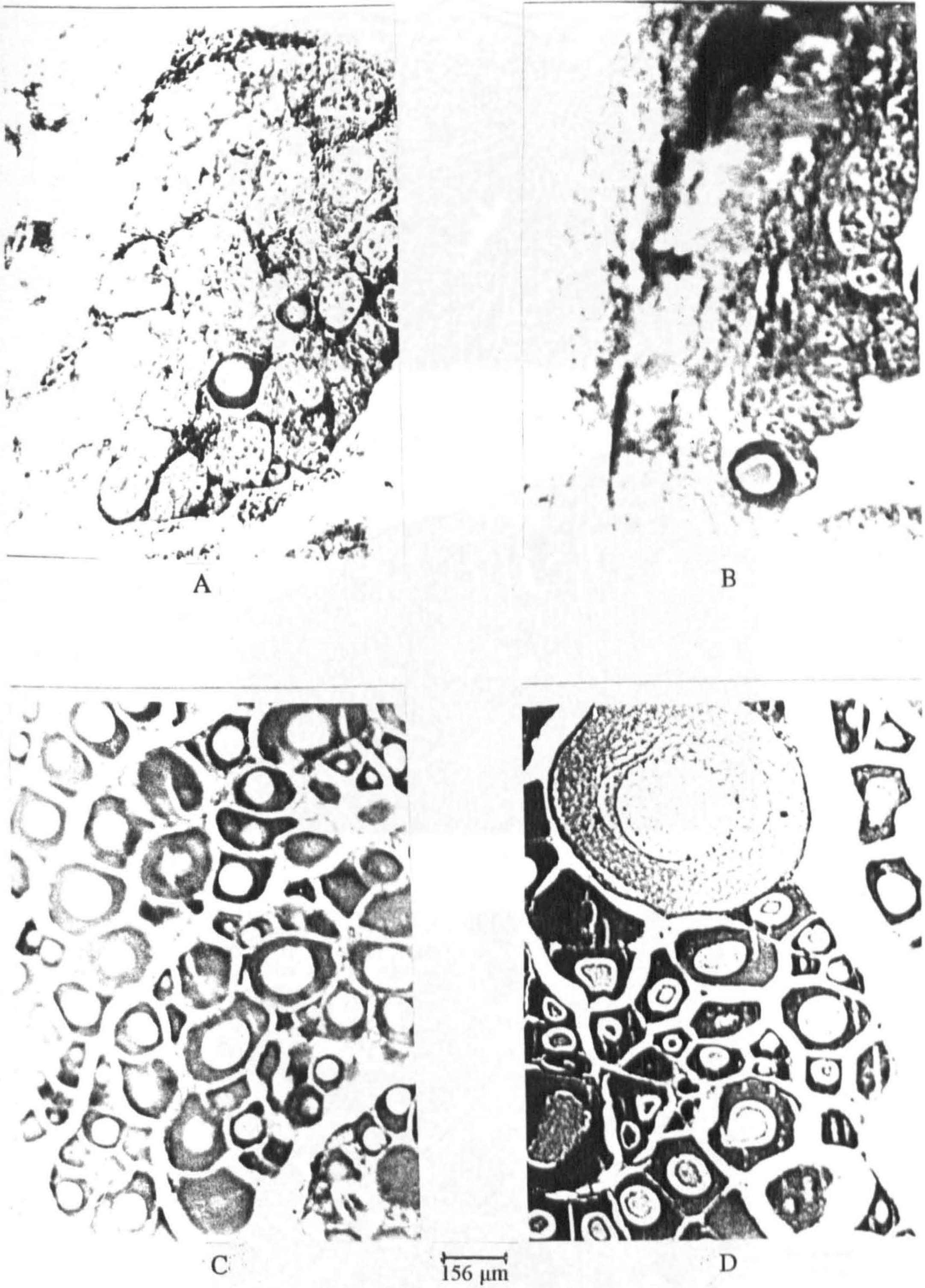


Figure 6.13 Histological section of ovaries in 4 groups of fish: A) diploid big-oui B) triploid hybrid C) diploid gynogenetic and D) *C. macrocephalus*

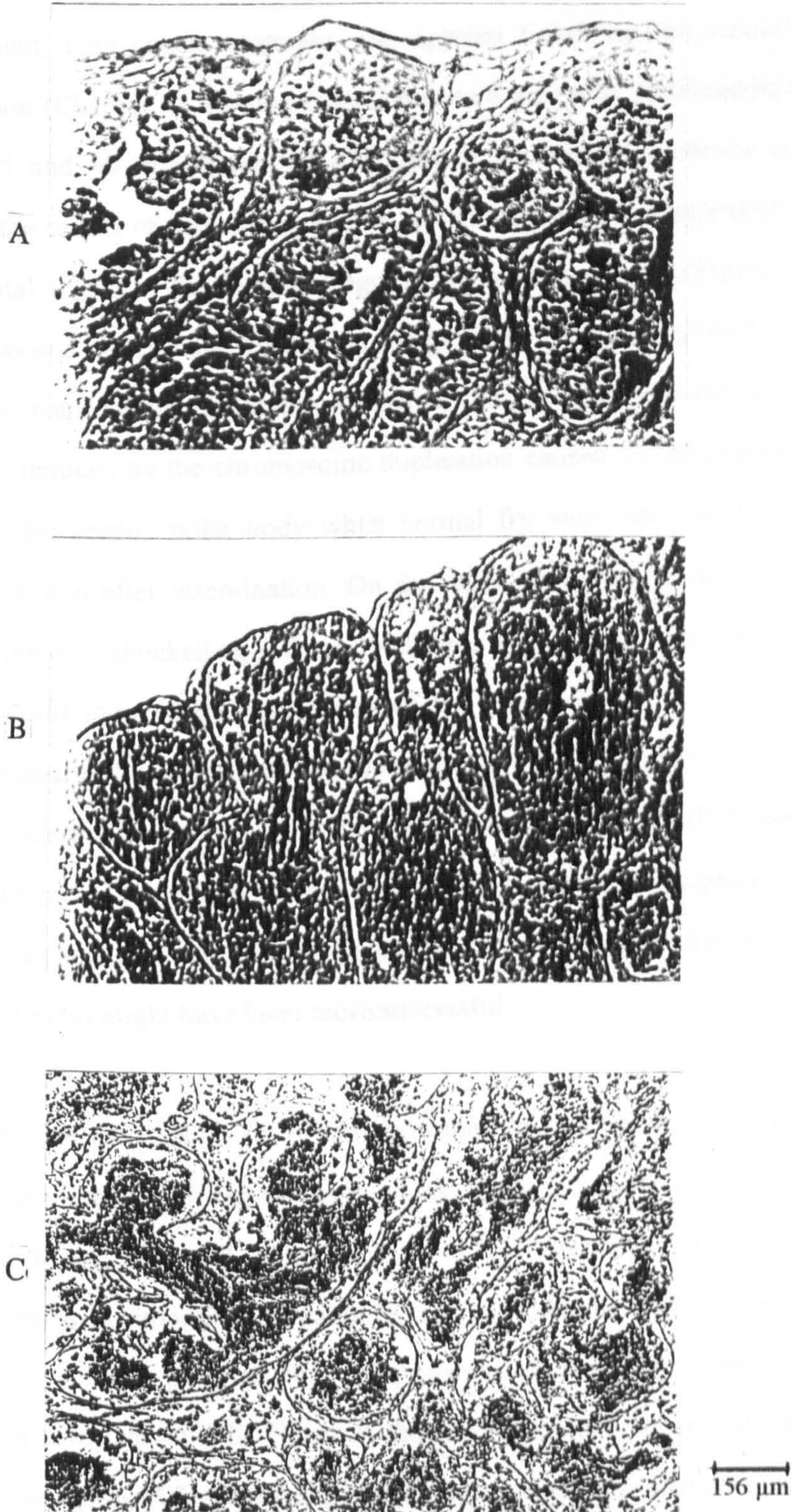


Figure 6.14 Testicular section of 3 groups of fish: A) diploid big-oui B) triploid hybrid and C) *C. macrocephalus*

In this experiment, cold shock treatment was applied following the results of triploidy induction (Chapter 5), starting from 2 min to 6 min after fertilisation and with 15, 20, 25 and 30 min durations. Changes in survival of embryos were correlated with the results of triploidy induction. From microscopic observations of the developmental stages of the fertilized eggs of *C. macrocephalus* (Figure 2.8, Chapter 2) the second meiosis and the first cleavage were ~10 min and 30~35 min after fertilisation, respectively. Therefore, almost all the gynogenetic diploids were considered to be induced by the chromosome duplication caused by the prevention of formation of the second polar body when normal fry were obtained by cold shock during 2~6 min after insemination. On the other hand, the fry which were produced from the cold shocked eggs from 30~35 min after insemination might be gynogenetic diploids induced by suppression of the first mitosis. However, cold shock treatment used in this study was not successful in the suppression of mitosis, since viable fry were scarcely obtained. This suggests that cold shock treatment may not be suitable for suppression of the first cleavage in fish, as suggestion for the red sea bream (Sugama *et al.*,1990). Alternatively, suppression of karyokinesis ratter than cytoclinesis might have been more successful.

In order to duplicate the haploid set of chromosomes of the egg nucleus, several different methods such cold shock, heat shock, high pressure and chemical treatment have been employed (Thorgaard, 1983). Onozato (1984) induced gynogenetic diploids in salmonids by using hydrostatic pressure and observed the disappearance of spindle poles during suppression of the first mitosis. The disappearance of aster and spindle fibers at the second meiosis when cold shock was applied in the loach (*Misgurnus anguillicaudatus*) eggs was also reported by Oshiro (1987). Therefore, the mechanism of diploid gynogenetic induction might involve the destruction of the division apparatus such as spindle fibers or spindle poles cause by physical shock. In this study, the time after fertilisation of 4 min and 15 min duration at fixed 2° C cold shock gave the highest survival rate of

gynogenetic diploids. At this time, the developmental stage of fertilized eggs was the anaphase of the second meiosis, separating the daughter chromosome sets. This result shows that the exact time during anaphase of the second meiosis may be an important key in inducing the formation of the diploid set of chromosomes by cold shock. The optimum time after fertilisation of cold shock applied to induce gynogenetic diploids in this study appeared to be the mid to late anaphase of the second meiosis of fertilized eggs in this species.

Fujioka (1993) produced gynogenetic diploids of honmoroko (*Gnathopogon caurulescens*) by cold shock treatment with UV irradiated sperm of nigorobuna (*Carassius carassius grandoculis*) and studied cytology in fertilized eggs. He reported that the egg showed the metaphase of the second maturation division at the time of insemination, and advanced to the anaphase by 5 to 7 min after insemination. The extrusion of the second polar body was observed 10 min after insemination and the metaphase of the first cleavage and the prophase of the second cleavage were seen 40 and 60 min after insemination, respectively. He also suggested that cold shock treatment (at 0° C for 40 min 3~7 min after insemination) is a practical method for induction of gynogenetic diploids in this species, caused by prevention of the formation of the second polar body. A similar result was reported in the production of gynogenetic diploids in loach (*Misgurnus anguillicaudatus*) by Oshiro (1987).

The gynogenetic diploid produced by the suppression of the first cleavage (mitotic gynogenesis) is considered to be more useful than the meiotic gynogenesis for the fixation and establishment of a new race of fish in aquaculture (Taniguchi *et al.*, 1988). The induction of homozygous diploid gynogenetics in zebra fish, *Brachydanio rerio*, in an attempt to produce cloned lines was demonstrated by Streisinger *et al.* (1981). Subsequently this technique has been applied to other species (Chourrout, 1984; Onozato, 1984; Purdom *et al.*, 1985; Ijiri, 1987; Krasznai

and Marian, 1987; Nagy, 1987; Taniguchi *et al.*, 1988; Sugama *et al.*, 1990; Komen *et al.*, 1991; Fujioka, 1993). In fact, while mitotic gynogenesis has been applied in many species, until recently cloned lines have only been reported in zebra fish (Streisinger *et al.*, 1981); medaka (Ijiri, 1987); common carp (Komen *et al.*,1991a) and Ayu (Han *et al.*, 1991). Cloned lines are potentially valuable products for selective breeding programmes and improvement of broodstocks.

In determining sex ratios of gynogenetic diploids at 3-4½ months of age in this study, a major effect of gynogenesis was the presence of 100 % female sex while the control pure *C. macrocephalus* produced the expected 1:1 ratio of sexes, The diploid big-oui hybrid performed consistently with the ratio of male 47.64 % : female 52.36% although gonad development seemed to be retarded (see Table 6.6). The sex ratios of mitotic gynogenetic diploids was reported as nearly 50:50% functional males and females in tilapia, while the meiotic group was nearly 100% female sex (Hussain, 1992). In the Ayu, *Plecoglossus altivelis*, Taniguchi *et al.* (1990) also observed a nearly 50:50% proportion of males:females in mitotic gynogenetic progeny. This frequency of mitotic males is consistent with the ratio of male: female = 46.7:53.2% in common carp where the mitotic males were homozygous for a recessive mutation (mas-1) in a sex determinating gene which induces a testis or an intersex gonad in XX offspring (Komen, *et al.*, 1988). Komen *et al.* (1991a) produced hybrid clones between homozygous females and XX mitotic gynogenetic males of common carp. Komen *et al.* (1991b) reported that heterozygous offspring of F_1 hybrids showed reduction in morphological variation compared to homozygous clones. Streisinger *et al.*, (1981) induced homozygous gynogenetic diploid inbred lines in zebra fish. They found variation in sex ratios among the inbred lines, which they suggested was not consistent with either a simple female homogametic or female heterogametic system but possible autosomal sex-determining genes or due to environmental effects.

In *Clarias gariepinus* females were found to be heterogametic (ZW) and males homogametic (ZZ) by Ozouf-Costaz *et al.* (1990). Teugels *et al.* (1992a) reported that a ZW heteromorphic pair is found in all female hybrids of female *Heterobranchus longifilis* x male *C. gariepinus* and female *C. gariepinus* x male *H. longiifilis* karyotypes while the ZZ chromosome pair in male hybrids is similar to that found in male *C. gariepinus* and in male *H. longifilis* specimens. From this evidence, therefore, in *C. macrocephalus*, males might have been supposed to be homogametic (ZZ) and females heterogametic (ZW). The sex ratio results from this study are suggestive of a homogametic female (XX) system. Clearly, further investigations of the sex determination system in this species (via e.g.hormonal sex reversal of gynogenetic diploid and crossing to normal females; or hormone sex reversal of normal fish and crossing to the opposite phenotypic sex and progeny testing) are required.

In this study, the experiment on mitotic gynogenesis was unsuccessful, at least partly due to a limited period of study, but the primary investigation has shown further potential in this field. Diploid gynogenetics are known to be useful in aquaculture for the production of inbred lines or monosexual broods. Therefore, further research is needed to characterize the gynogenetic diploids and to establish the optimum treatment for producing homozygous gynogenetic diploids by suppression of the first cleavage (mitotic gynogenesis). An efficient way of producing clonal lines in this species would be useful for future research.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

7.1 Aquacultural benefits attempted from this research

Freshwater catfish especially *Clarias spp* are widely distributed in southeast and south Asia and Africa. They exist in a variety of habitats from brackish water in estuaries and mangrove areas to fully freshwater rivers and lakes. Recently, they have become a popular food fish and subsequently have become a popular species for culture because they can be spawned artificially, grow very fast, can tolerate farming conditions and are easy to culture.

In Thailand, *Clarias* catfish are now one of the most important cultured fish. The two most economically important native species are *Clarias macrocephalus* and *C. batrachus*. Another species of African catfish, *Clarias gariepinus* was introduced in 1987, this species was distributed and cultured in the northeast region and some provinces in the central region. Furthermore, the breeding of the big-oui hybrid catfish was achieved by using male *C. gariepinus* and female *C. macrocephalus*. This hybrid gives faster growth than *C. macrocephalus*, has high resistance to environmental conditions (similar to *C. gariepinus*) and intermediate morphological characteristics and meat quality compared to the parental species. The big-oui hybrid has become increasingly important and generate high demand in the markets. In the culture ponds,

this hybrid can grow up to marketable size within 3 months so that the farmers can produce at least 3 crops anually, an advantage in animal protein production.

The growing awareness that fish is a healthy source of protein has further driven demand for more fish worldwide. The increasing importance of cultured fish as a food fish has prompted considerable research on genetic improvement of the commercially important species. Much of this work has concentrated on the development of genetic manipulation techniques with varied applied objectives. The most commonly attempted rationale has been the production of sterile offspring to negate the problem of precocious sexual maturity and unwanted reproduction in culture. This was first successful in hybrid grass carp x big head carp (Marian and Krasznai, 1978 and Krasznai *et al.*,1984b). The justification for sex control in fish stocks introduced from a foreign territory may have several bases. The primary consideration may be to prevent reproduction and thereby remove the potential for becoming naturalized or established within the new range. Thus, eliminating unwanted reproduction through sex control is primarily a security measure, either to allow evaluation of an introduced fish in an ecologically safe manner or for its ultimate utilization (Shelton, 1987). From the perspective of transplantation or introduction, reproductive control may be desired to prevent gene pool contamination through hybridization with conspecific races or strains. Shelton and Smitherman (1984) discussed how to introduce fishes in warmwater aquaculture with considerations for application of sex control.

Sex control may be very beneficial in a production system, as growth may be enhanced by reduced competition from progeny, taking advantage of sexually dimorphic

characters, or increasing food conversion efficiency through diversion of gonadal energy demands into somatic elaboration. All are benefits to aquaculture through sex control and may be considered for research. Donaldson and Hunter (1982) and Yamazaki (1983) reviewed control of sex in fishes. Within the perspective of sex control through genetic manipulations, it can be considered that the primary mechanisms are monosexing or sterilization. Stocking only one sex provides considerable security against reproduction, but the monosexing effort must be totally effective. Interspecific hybridization may produce highly skewed sex ratios but rarely entirely monosex populations. Steriod induced sex inversion, temperature regulation or pH influence are physiological alterations that may have genetic implications. Both hybridization and hormone sex inversion are used in aquaculture of tilapias where a level of 90-98 % effectiveness has been achieved (Hulata *et al.*, 1983 and Mair, 1993). Hormone induced sex inversion may be a functional component for production of broodstocks, which are used for spawning to produce monosex offspring. There is considerable variability in sex determination among fishes which may affect the precision of this procedure (Shelton, 1987). Monosexing can be appropriate for introduced (exotic) species but has limitations for transplanting species, depending on the relationship of resident species. Closely related species with similar reproductive biology present the opportunity for hybridization. Considering the selective disadvantage for successful reproduction of hybrids in a natural community, Chevassus (1983) suggested this may have minimal significance. However, if transplanted species overlap sibling subspecies, then inbreeding will occur and result in gene pool degradation. Thus, under appropriate conditions, some approaches to the production of

monosex populations might be considered for stocking introduced fishes but not transplanting species within their natural range (Shelton, 1987).

Sterility should be the method of best choice in sex control. Genetic-based sterility involves polyploidization, especially triploidy, which has been achieved through some intergeneric hybridizations or by chromosome manipulation (Stanley, 1982). Hybrid reproductive potential may vary from complete sterility to apparently normal fertility (Chevassus, 1983). Concern may still be warranted because of differential effects between sexes. Triploid females usually have poorly developed ovaries with only scattered ova, while males have more extensive morphological development but with spermatogenesis usually not culminating in functional spermatozoa (Gervai *et al.*, 1980 and Wolters *et al.*, 1982b). The differential development and possibility of some viable gametes demands additional investigation. A more involved extension of this approach would be to combine techniques to produce triploid monosex populations (Shelton, 1987).

7.2 General discussion on genetic approaches using manipulation and related techniques in this study.

In discussing various means of achieving sex control, Shelton (1987) mentioned some for completeness despite their perceived impractically and others are included because of their potential importance as components of more involved but pertinent systems. He generally categorized these into methods that involve a direct treatment or manipulation and those which are preliminary to developing a broodstock for use in a breeding programme. These methods may have questionable productivity, quality control and a

mixture of physiological and genetic elements. These approaches seek to produce populations of single sex or sterile fish through some form of manipulation for each individual, absolute success cannot be expected since individuals that are not altered by the treatment usually are mixed within the populations.

Steroid induced sex inversion: the development of phenotypic sex in fishes is under genetic control but may be mediated by environmentally influenced physiological means (Hunter and Donaldson, 1983). Gonadal development in fishes passes through a labile indifferent phase prior to phenotypic expression as an ovary or testis. Steroids have been widely applied as an exogenous means of directing gonadal differentiation indepent of the genotype (Yamazaki, 1983). Exposure has been through immersion, via food or through implantation. Absolute monosexing through steroid induced sex inversion is not generally uniformly achieved (Shelton, 1987). However, since 100% monosex populations have not been consistently produced by direct steroid sex inversion, the application of sex control to an introduced fish for stocking in open waters cannot be considered as adequate to provide appropriate security.

Hybridization: A general phenomenon among fishes is the production of progeny with a range of moderately to highly skewed sex ratios (Chevassus, 1983). Skewed sex ratios have been produced from various interspecific hybridizations, most notable among tilapias, particular crosses have yielded nearly 100% male populations, which have utility for aquaculture (Pruginin *et al.*,1975). Interspecific hybrids are fully fertile, although reproductive behavior is logically altered, F_1 hybrid and back-cross with parental species are common. For aquacultural purpose, hybrid tilapias have also been

androgen treated in an effort to maintain advantage of hybrid vigor and come closer to attaining monosex population (Mair, 1993).

Gynogenesis: the development of an ovum without a paternal genetic contribution. The first application for gynogenesis is as a means of producing single sex offspring, assuming the involvement of only the maternal genome, which is of the homogametic sex. Functional homogamety has been demonstrated through gynogenesis in various species. Spontaneous gynogenesis through second polar body retention is rare, although the frequency can be increased by a variety of physical and chemical shocks at the appropriate time in retention to the polar body formation. The females produced are fully fertile, and therefore, reproductive control through monosex stocking will be compromised if prior mixed sex stocking has occurred. However, the main problem with many species is the low yield of diploids despite shocking to enhance production. Induced gynogenesis can also be achieved through suppression of the first mitotic division in the developing haploids. While this may be accomplished by judicious application of a late shock (Thorgaard, 1983), the success is lower than in early shock. One aspect of optimizing gynogenetic treatments that has not been adequately exploited is its value as an effective estimator of the most probable optimal treatment conditions for polyploidization. Optimizing diploid production by either early shock and late shock should correlate with the greatest yield of polyploids. Evalution is direct through counting of surviving diploids.

Polyploidization: manipulation of meiotic and mitotic events may produce polyploid individuals from ova that have been inseminated with genome-bearing sperm (Purdom,

1983 and Thorgaard, 1983). The sperm status is the main distinction between induction of polyploids versus gynogenetics. Gynogenetic induction regimen with reference to treatment, type and intensity of shock, time of application and duration can be readily optimized for a species by evaluating the yield of viable diploids, since the haploids die before swim-up. The analogous regime can be applied to polyploidization with anticipated optimal results simply by omitting the DNA-denaturing treatment of sperm. Evaluation becomes more complex since both diploids and polyploids are viable. Whether the sperm is from the same species as the ova donor or a related species will determine whether a polyploid species or a polyploid hybrid is produced, respectively. If an early shock is applied to increase the incidence of second polar body retention, triploid induction results. If shock is delayed until the period associated with the first cleavage, tetraploidy may result.

7.3 Implications of the present results in *Clarias* catfish culture

Like salmonids and tilapias, production of expectedly sterile hybrid catfish by genome manipulation techniques has attracted considerable attention in recent years. A few work has been reported on the effective optima of temperature shock and pressure shock in this species (Na-Nakorn, *et al.* 1993b; Volckaert, *et al.* 1994 and Ezaz, *et al.* 1996). The present study was carried out to identify treatment optima for triploidy induction and diploid gynogenetic induction by altering intensity, duration and timings of applications of cold, heat and pressure shocks and uses, in this instance, perturbation of meiotic division of eggs as an experimental model. Cold shock was found to be the most effective agent in induction responses while heat and hydrostatic pressure were

found to be less effective and to give low viability of embryos. Cold shock treatments have some advantages over the other agents and proved to be a very reliable method for triploidy induction in these species.

Triploids do not have normal gonadal development (Thorgaard, 1983), they have much lower gonado somatic indices (Lincoln and Scott, 1984) and they are functionally sterile because they produce aneuploid gametes (Allen, 1987). The result from this research confirmed that triploid big-oui hybrids have functionally sterile gonads. Hence, they might grow faster than diploids as they reach the age of sexual maturity because they may direct energy from reproduction to somatic growth. Sterility may also suppress some of the other undesirable phenomena associated with reproduction such as reduced appetite, reduced feed conversion efficiency, deterioration in flesh quality and postspawning mortality. Despite abnormal gonadal development, triploid big-oui hybrids showed external sexual differentiation similar to that of diploid hybrids. This is however of little relevance in Thai aquaculture because diploid hybrids already have reduced reproductive performance, and the culture period is not long enough for the fish to reach maturation anyway.

In comparision of growth between diploid and triploid big-oui hybrids, triploid hybrids were found to have similar growth to diploids. A few studies of the growth and condition of triploid adults have been reported (Swarup, 1959; Purdom, 1972; Lincoln, 1981b; Valenti, 1975 and Penman *et al.*, 1987b). The results are equivocal: some studies reported a slight growth advantage of triploid, whereas others found similar or even reduced growth of triploids compared with diploids (Penman *et al.*, 1987b). Ihssen, *et al.* (1990) suggested that the growth advantage of triploids is species

dependent, even for closely related species. Also, the growth advantage may be held only by females, not by males that develop pronounced secondary sexual characters and even sperm. The faster growth of females, if simply measured as total body weight at age, may be partly due to the greater accumulation of fat in the body cavity rather than the desired increase in muscle tissue (Lincoln and Scott, 1984). Triploid induction may improve the viability of interspecific hybrids (Chevassus, 1983; Scheerer and Thorgaard, 1989). Some hybrids such as rainbow trout x brown trout, which are almost completely nonviable, have much improved survival if triploidy is induced. The induction of triploid big-oui hybrid did not show improvement in this respect.

In generally, triploid hybrids are derived from the suppression of the second meiotic division and thus they possess two maternal haploid sets of chromosomes and one paternal set. This property makes it possible to design hybrids that have a higher proportion of the characteristics of one species relative to the other in one generation. If reciprocal triploid hybrids are also available, a variety of hybrids having different proportions of maternal and paternal characters can be produced. In diploids, such a rearrangement of parental characters is possible by backcrossing (if F_1 hybrid is fertile), and it would require two generations compared to one for triploids. All of these possibilities have been proven impossible events in big-oui hybrids.

Suppression of the second meiotic division to produce gynogenetic diploids and triploids has been much more successful than the suppression of the first mitotic division to produce mitotic gynogenetic diploids and tetraploids. However, even for meiotic gynogenetic diploids in this study, yields have been extremely variable and

often very low because their abnormalities and usually they died after yolk-sac resorption. Another complication affecting yields of gynogenetic diploids is that partially inactivated sperm, by contributing chromosome fragments to the gynogenetic diploids, may lower the viability of those diploids. This phenomenon is primarily associated with ionizing radiation, not with UV-irradiation (Chourrout, 1987). The method of sperm inactivation may affect yields, but it does not seem to be as critical as the other treatment factors because very low yields of gynogenetic diploids are often accompanied by high yields of the corresponding haploid. Sperm inactivation and vitality effects can be separated from meiotic or mitotic treatment effects by incubating haploid and diploid controls alongside the gynogenetic diploids. If the frequency of diploids among the haploids is larger than frequencies expected for spontaneous gynogenetic diploids, the sperm have not been completely inactivated and irradiation dosages should be increased. If, on the other hand, the proportion of nondeveloping eggs is larger among haploids than among the diploid controls, the treatment of the sperm may have been too severe, causing loss of sperm vitality, and irradiation dosages should be decreased. For UV-irradiation, diploids other than spontaneous gynogenetic diploids have been found only very rarely among haploid controls, and the proportion of developing haploids is usually similar to that of diploid controls. So that, UV-irradiation effectively inactivated sperm without significant loss of sperm vitality (Ihssen *et al.*, 1990).

7.4 Further hybridization and genetic manipulation studies in *Clarias* catfish

In future, genetic research on *Clarias* catfish has many potential applications in the aquaculture of these fish. Studies and development of hybridization and genetic manipulation methodologies to produce improved stocks have just begun to be investigated. More trained scientists are essential in the future to develop better commercial strains of broodfish and produce suitable fry for economical farming conditions in Thailand and Asian countries. Apart from the results of *Clarias* catfish hybridization and genetic manipulation research of previous and present work, there are some other interesting tropics for further investigations as follows:

1) Undertaking of other interspecific hybridizations: There seem to be multiple aspects for studies on interspecific hybridization, especially among *Clarias* catfish. The main motivations are to obtain a better fish for commercial purpose and recently also to intensify aquaculture. A 'better fish' may be obtained by:

- Hybrid vigor, i.e., the hybrids perform beyond the range of their parent species and their performance is of value for intensification of aquaculture.
- A combination of traits, for instance, high tolerance of one species may be combined with rapid growth of another.
- Sterility to prevent growth reduction in connection with sexual maturity, to obtain population control when stocking natural waters with introduced species or to prevent gene introgression between natural and culture stocks.

The big-oui hybrid is an example of valuable traits combining the good growth rate of *C. gariepinus* with the good meat quality of *C. macrocephalus*, which together made the hybrids very productive in pond culture. Another interesting, although not yet fully investigated application is artificial hybridization between *C. gariepinus* x *C. batrachus* and artificial hybridization between *C. macrocephalus* x *C. batrachus*. Further research could be pointed to these topics.

2) Sterile fish: The induction of triploid big-oui hybrid did not show improvement of survival and triploid big-oui hybrids were found to have similar growth to diploids. As triploid big-oui hybrids have been found to be functionally and reproductively sterile, such sterility of triploid can be of interest in itself, quite apart from other physiological and morphological considerations. Therefore, it is necessary to study the behaviour of the triploid hybrid in comparison with the diploid hybrid. If mature triploid hybrids were completely sterile, then the use of triploidy could guarantee lack of genetic interaction by escapees from farms (but would this be feasible in aquaculture: yields, growth rates, food ultilization, etc). Although maturation of big-oui hybrid in culture or in the wild is not considered a problem at present, this study has shown that back cross hybrid can be performed and it is therefore possible that breeding big-oui hybrid may become a problem in the future. This technique of sterilization can be applied in *Clarias* catfish or other important culturable species, where control of natural reproduction is sometimes essential for the benefit of their aquaculture. Sterile fish also permit more precise control of the number of fish per tank or pond until harvest and hence better rearing conditions.

Triploidy induction in the reciprocal cross hybrid and the back cross hybrid are another interesting investigation, aimed to improve the survival of these crosses and growth performance. The preliminary experiment of Mohidin (1995), on induction of triploid reciprocal cross hybrids was carried out following the techniques developed from this study. His results showed that triploid reciprocal cross hybrids can be produced even though the survival rates of fry (at 3 day old) were very low. The highest percentage of triploidy was 57.1% when cold shocking (4°C) was applied at 3 min after fertilisation and duration of 20 min.

3) Tetraploidy induction: The induction of tetraploidy in rainbow trout and the viability of tetraploids has been reported by Chourrout *et al.*, (1986). Induced triploid production of big-oui hybrid has limitations in low survival rate and body deformation. Further research is therefore needed to develop viable tetraploid broodstocks for using in crosses between diploid x tetraploid, to produce subsequent large-scale generations of hybrid triploids. The preliminary study in cold shock techniques developed for inhibition of mitotic cleavage of eggs may be useful to induce viable tetraploids in big-oui hybrid.

4) Further study of induction of mitotic gynogenetics and production of their clones in *C. macrocephalus* is necessary. Such clones are genetically uniform, a reduction in phenotypic variation and increased or superior developmental stability is therefore expected (Komen *et al.*, 1991b). It is not out of the question, however, homozygous clones may have the reverse effect and reduce developmental stability. Interesting research with clones as standardised animals could be carried out on heritability,

immunoresponse, disease resistance and sex differentiation studies. Clones could be used as controls in selection experiments particularly in commercial testing. Further research could be initiated to develop the technique for production of clones and commercial *Clarias* catfish strains. Research on clonal lines could be expanded involving studies on the growth, other phenotypic traits and investigation of sexual development of the outbred and inbred clones.

5) Monosex culture of *C. macrocephalus* female broodstock for big-oui hybrid production: the big-oui hybrid has become a popular cultured fish and is now the single biggest freshwater fish product in Thailand. *C. macrocephalus* is difficult to produce in large numbers and high demand of this species for broodstock to produce hybrids has led to a shortage of this species. The results from this study indicate that male *C. macrocephalus* cannot be used in hybrid production because the reciprocal cross (female *C. gariepinus* x male *C. macrocephalus*) does not survive although genetically it appears to be similar to the big-oui.

There are a number of potential methods for monosex female culture in such situations, including hormone treatments or 'indirect' monosex methods via genetic manipulation of broodstock. These can used as specific breeding tools in addition to the more general broodstock management and selection programmes which should be ultilised in *Clarias* catfish culture. Work in Thailand had been experimenting with 'direct' methods, especially, hormonal feminization of *C. macrocephalus* fry as reported by Pongthana *et al.*, (in press) who had success at producing 100% females without any further alteration in culture practices. It may be concluded that hormonal feminisation

techniques can potentially be applied in present situations in *Clarias* culture in Thailand. For the long term solution, 'indirect' monosex culture via genetic manipulation, where the fry for broodstock production receive no steroid treatment, would be used to produce fish. This relies on an understanding of the sex determination system in the species concerned. Further research work aimed at investigating the sex determination system in *Clarias* species will be necessary to solve this problem. In this study, 9 batches of 100% female gynogenetics (153 individuals) were produced suggesting that *C. macrocephalus* should have the XY male/XX female sex determination system. Following with gynogenetic induction incorporation with hormone sex-reversed to produce homogametic males are thought to be useful in crossbreeding experiments to produce all female monosex populations where female fish are more preferable as in this case. Mirza and Shelton (1985) reported the results of the intraperitoneal administration of methyltestosterone-silastic implant to gynogenetic silver carp. Examination of the gonads of 17 implanted fish showed that 13 fish had testes, one intersexual gonads and three had gonads with no germ cells. Mohidin (1995) investigated hormone sex reversal of gynogenetic diploid offspring using the gynogenesis techniques developed from this study. He showed that phenotypic males were obtained from all treatment groups of oral administration of the synthetic androgens (11β-hydroxy-4 androsten-3,17-dione and 17-hydroxy-17α-methylandrostan-17β-ol-3-one) at different dose levels (10, 50 and 100 mg/kg feed) for duration of 4 weeks. This compared to all female untreated gynogenetics. Progeny testing of such fish will provide further information on the sex determining system in this species.

6) DNA fingerprinting: The techniques have been successfully applied in humans and are becoming increasingly important and popular for fish. Research in this field using DNA probes will open up new avenues to analyze and estimate the degree of inbreeding associated with both meiotic and mitotic gynogenetics as well as of natural populations. DNA probes have now been isolated that hybridize to a single locus and ultimately these locus-specific probes may be of value in establishing linkage to genes affecting important traits such as growth rate and disease resistant.

7) Transgenic fish: In the past 10 years, gene transfer technology was explored in various animal species. The transfer of cloned genes to the embryos of mice and several domestic animals has been achieved by microinjection of hundreds or thousands of copies into the male pronucleus. Some of the integrated genes are expressed, and some of the transgenic mice obtained by injection of growth hormone genes grew rapidly (Chourrout, 1987). The objectives of producing transgenic fish are particularly focused on growth enhancement and disease resistant. The first publications in production of transgenic fish came from the research group working in the People's Republic of China (Zhu *et al.*, 1985; 1986, cited by Woodwark *et al.*, 1994). They introduced a DNA construct consisting of the coding sequences of the human growth hormone gene fused to a mouse metallothionein promoter into cyprinid and loach species. Chourrout *et al.*, (1986) tried to inject 20,000,000 copies of a plasmid containing the human growth hormone gene into rainbow trout egg cytoplasm. They suggested the proportion of positive embryos was higher when linear plasmids were used instead of circular ones. The similar results were reported by Maclean, *et al.*, (1987), they injected 10^6 copies of a cloned DNA sequence consisting of the mouse metallothionein gene

promoter spliced to a genomic copy of the rat growth hormone gene into fertilised eggs of rainbow trout. The original gene construct used a mouse metallothionein promoter controlling a rat growth hormone gene. This mammalian constructs have had no or modest effects on growth in trangenic fish, prompting the development of fish gene constructs with improved effects on growth performance. However, public concern over the use of DNA from non homologous sources makes it desirable to develop constructs from as close to homologous DNA is now practical. Devlin *et al.* (1994) microinjected linear pOnMTGH-1 DNA where all genetic elements were derived from sockeye salmon and consisted of the metallothionein-B promoter fused to the growth hormone gene. They found that 6.2 % of the individuals surviving to one year and had modal weight as controls but contained many larger individuals which retained pOnMTGH-1 DNA in their fin tissue, indicating that the presence of this gene construct was responsible for the growth enhancement. The gene transfer technique may be very promising for the genetic improvement of cultivated species, fishes are being an excellent material for such investigations, so, much effort is likely to be spent in gene transfer in the future.

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APPENDICES

APPENDIX 1

A) Examples of salines used for dilution of fish sperm without activation:

	Amount per saline (gl^{-1})			
Chemical	Modified Cortland's Saline	Modified Fish Ringer's	*Clarias* extender # 1	Normal Saline
NaCl	1.88	6.50	5.16	8.50
KCl	7.20	3.00	1.64	-
Chemical	0.13	0.15	0.14	-
NaCl	0.36	-	0.36	-
NaHCO3	1.00	0.20	1.00	-
MgSO4	0.11	-	0.11	-
Glucose	1.00	-	-	-
Fructose	-	-	1.00	-
pH	7.0	8.0	7.3	-

Remarks:

Modified Cortland's saline : chemicals should be added to water in order shown, with each chemical being allowed to dissolve completely before the next is added (to avoid precipitation). Species recommended : tilapia, *Clarias* catfish.

Modified Fish Ringer's saline. Species recommended : tilapia.

Clarias extender # 1 saline. Species recommended : *Clarias* catfish.

Normal saline; 1: 3 or 1: 9 sperm: saline dilution for common carp; 1: 99 for *Puntius gonionotus*. Species recommended common carp, *P. gonionotus* , etc.

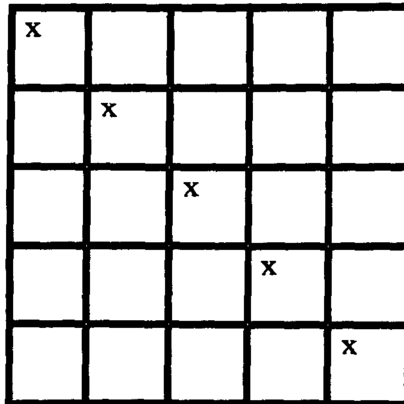
B) Procedure for counting sperm concentration:

1. Strip sperm, collect into a tube and refrigerate.

2. Dilute with saline (see Chapter 2, section 2.6). The dilution used will need to be determined by trial and error; it is necessary to produce a diluted sample which can be scored easily with the haemocytometer. For *C. gariepinus*, 1: 50-1: 600 is appropriate, depending on initial concentration.

3. Set up the haemcytometer with the cover slip in place. Introduce the diluted sperm from the side, without flooding the side channels of the central section.

4. Haemocytometers vary in the size of their cells : I use a 1/20 x 1/20 x 1/10 mm (1/4,000 mm^3) cell size convenient. The arrangement on this type is as shown below



Each square is subdivided into 16 smaller cells (4 x 4). Each of these smaller cells has a volume of 1/4,000 mm^3.

The sperm suspension is left for about 5 minutes. This allows the sperm (non motile at this stage) to settle down onto the surface of the grid, making them easier to count.

5. Counting all the sperm in each cell from the 5 squares marked "x" above gives a total of 80 cells (4 x 4 x 5). This is a good sample from which to obtain a mean

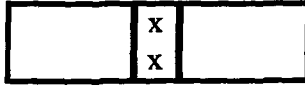
(Average number of sperm heads / 80 cells). As this is the number of sperm per $1/4{,}000\ mm^3$, the concentration in the undiluted sperm sample can be obtained as follows :

Concentration (sperm cells ml^{-1}) = Average mean x 4,000 x 1,000 x dilution factor

For example : ideal sperm concentration = 2.5×10^8 cells/ml.

To obtain such a concentration, we have to follow this procedure :

-collect sperm
-check sperm motility post activation by adding 5 µl of diluted sperm to 50 µl of water in a microcentrifuge tube and then rapidly placing a drop of the mixture on a glass slide (ready set up before) for microscopic examination. Only diluted sperm samples with a motility score of 9-10 after activation were used.
-take 490 µl of Modified Cortland's saline, place into the first microcentrifuge tube.
-take 10 µl of diluted sperm and add to the same microcentrifuge tube: this will make a concentration of 1: 50
-take 90 µl of Modified Cortland's saline, place into the second microcentrifuge tube.
-take 10 µl from the first microcentrifuge tube and add to the second microcentrifuge tube: this will make a concentration of 1: 500
-take the sample from the second tube and place onto a haemacytometer.



-wait for 5 minutes to allow the sperm to settledown, until sperm stops drifting.

-count sperm heads in each small cells (4 x 4 cells) from the 5 squares marked "x" as above (80 small squares in total)

-find out the total number, calculate the average mean, eg. the total count = 200 sperm heads.

Average mean = 200/80 = 2.5 sperm heads

Concentration = 2.5 x 4,000 x 1,000 x (50 x 10) cells /ml

= 5×10^9 cells /ml

Concentration required = 2.5×10^8 cells / ml

-if the amount volume of diluted sperm required = 1,000 μl

The amount of sperm volume = $1{,}000 \times 2.5 \times 10^8 / 5 \times 10^9$ μl

= 50 μl

-mix 50 μl of undiluted sperm with 950 μl of Modified Cortland's saline to meet the final concentration of 2.5×10^8 sperm cells/ ml

The dilution factor = 50 : 1,000 = 1 : 20

APPENDIX 2

KARYOLOGICAL EXAMINATION

A) Tissue preparation

Colchicine (0.5 % stock solution) is required to block cell division at metaphase. Take 1 ml of the stock solution and add 20 ml of incubator water.

Add the required number of embryos to the colchicine solution and leave for a minimum of 4 hours. The embryos can be left for longer providing they are still living.

Drain off the colchicine and add 0.7 % NaCl, remove the yolksac from the embryos and put into distilled water for 20 minutes. This will swell the embryonic cells.

Finnally put the embryos into an ethanol / acetic acid 4: 1 mixture where they can be stored for up to 30 days at 4° C.

B) Chromosome preparation

Set up a hot plate at a temperature of 45° C.

Wash microscope slides in ethanol. One slide will be required for each embryo.

Handle slides by their edges only.

Add 5 drops of acetic acid (50 %) into each hole. One hole will be required for each embryo.

Place the embryo into a watch glass. Remove one of the embryos and dry the fixative off using a tissue and place into the first hole (start from left hand side).

Note the starting time and begin to "shake" the embryo in the acetic acid for approximate 45 secounds (the embryo can be minced for 1 minute with glass rod or scraped by using fine forceps to dissociate epithelial cells). The acetic acid breaks down cell bonds and the "shaking" should release tissue into the solution. Remove embryo.

Leave the tissue and cell in the acetic acid for 12-15 minutes.

Remove the acetic acid and tissue solution using a micro-pipette dropper and drop solution into the heated slide. Leave for approximate 10 seconds, then draw some of the solution up. Leave the slide for a further 10 seconds and then draw more liquid up and so on. More than one set of ring can be done on each slide.

C) Staining slides

Take 4 ml of Giemsa stain and put into a staining glass. Add 36 ml of 0.01 M phosphate buffer: the buffer is freshly made up from 2 stocks namely 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4 (6.8 g / 100 ml distilled water and 17.9 g / 100 ml distilled water respectively). Mix 6.26 ml of 0.5 M KH_2PO_4 and 4.56 ml of 0.5 M Na_2HPO_4, and dilute with distilled water to 500 ml.

Place the slides back to back into the staining glass and leave for 20 minutes.
Wash slides in distilled water and leave to air dry.
Then put the slides into Xylene and leave for 10 minutes.
Air dry for 1-3 hours.

Mount the slides using 3 drops of DPX mountant.

Leave to dry and store.

Procedure for photographing through a Leitz Orthomat photomicroscope

Loading the Leitz Orthomat 35 mm Camera

1. Remove cassette from camera back.
2. Open cassette.
3. Push film lead under the clamping spring of the spool, emulsion side up.
4. Pull out rewind key and insert 35 mm cartridge.
5. Turn film spool by knurled knob in direction of the cartridge until the film becomes taut and both sides of the perforation are engaged in the sprockets.
6. Close cassette.
7. Set frame counter (N.B. This counter indicates the number of unexposed frames).
8. Set the ASA/DIN and type of film on the back of the cassette.
9. Replace cassette in camera back so the counter is visible through the window at the RHS of camera.
10. Lock camera back.
11. Set ASA/DIN on control panel (N.B. Separate knobs for B/W and color film).
12. Press ‘flash synch’ button.
13. Press ‘expose’ button 2-3 times.
14. Press ‘flash synch’ button again - light will go off.
15. The film is now wound on ready to take pictures.
16. When all of the film has been exposed (indicated by a buzzing sound) press the ‘film reset’ button at the side of the camera.
17. Open camera back and remove cassete.

18. Press button on the side of the cassette and rewind film.

19. Remove film from cassete.

Operating instructions for the Leitz Orthamat photomicroscope

1. Switch on microscope light. Turn up brightess unti needle is just below red line.

2. Switch on control box.

3. Insert clean slide and select field.

4. Open camera back and remove cassette (See separete instrucyion for loadind camera.

5. Locate focusing screen and magnifier in camera back.

6. Press 'shutter open' button on control box.

7. Focus image at film plane i.e. on the focusing screen.

8. Adjust each eyepiece separately to suit eyes (N.B. Focus on infinity not on the specimen or graticule. This prevents accommodation by the eye).

9. Press 'shutter open' button to close shutter. Remove focusing screen.

10. Open aperture diaphragm.

11. Close field diaphragm.

12. Insert or remove supplimentary condenser as necessary.

13. Rack condenser up/down to focus.

14. centre the spot of light.

15. Open field diaphragm until just clear of field.

16. Close aperture diaphagm until exit pupil reduced by (N.B. The condenser must be refocused and centred each time the objective lense is changed.

17. Recheck focus.

18. Check orientation of camera.

19. Check framing.

20. Select filter and place in light beam.

21. Load and relocate film cassette. Wind on film for 2/3 frames by pressing the 'expose' button.
22. Check expose and then shoot.
23. When film completely exposed (indicated by a buzzing sound) press 'film reset' button on the side of the camera.

Developing 35 mm black&white film

Put on red light in corridor.

Paterson spool must be dry

Set waterbath in sink at 20°C

Turn on extractor fan

Arrange dismantled paterson tank on bench with scissors and spool

Put light off (in total darkness)

Remove film from spool

Unwrap film and cut both ends straight with scissors

Wind film on to paterson spool

Assemble paterson tank

Put light on

Use gloves

Add developer to paterson tank; at 1:1 0.1 stock strength 5.5 mins

Put on white lid, shake occasionally (times vary according to dilution; check bottle)

Set stopwatch

Remove developer to bottle; and rinse 3 times with water at 20°C

Add fixer and shake occasionally for 3-4 mins

Remove fixer to bottle (can be used several times)

Remove film from tank

Place in running water in waterbath at 20°C for 15-20 mins

Turn water off and add 10 ml of photoflo and leave for a few mins

Suspend film in drying cupboard and leave for an hour

Rinse paterson tank well and place in bottom of drying cupboard

When negatives dry cut to size and place into plastic strips

To make contact prints

Put red light on in corridor

Put on water to processing machine (turn tap 3 times)

Turn knob to run possition (it takes 30-45 mins for machine to warm up)

Make contact prints of all negatives:-

Raise enlarger to highest position (spring mechanism)

Set aperture on enlarger to 8 or 5.6 (aim for exposure time of 5-8 s)

Switch on enlarger

Left hand side	Right hand side
Exposure time	Paper grade
	Contrast 1-5

Put on yellow light and main light off

Place large sheet of photopaper on glass press

Lay negatives on top of paper and close

Set exposure time and grade (write details on the back of the paper)

Switch on

When exposure complete put print shiny side down into processing machine

To make enlargements

Lower enlarger

Check light source is the small light box (it becomes very hot)

Place negative in holder

Press focus button on control and focus enlarger onto white board; adjust fine focus so that the papers grain is in focus with the magnifier; adjust sides to mask light and center frame

Decrease aperture setting	Number for darker prints
Increase exposure time	Number for darker prints
Increase grade setting	To increase contrast

Put on yellow light and main light off

Take out one sheet of paper (close box)

Write settings on back

Place paper in frame (shiny side up)

Press exposure botton

Take paper and place shiny side thinways into machine

Repeat and adjust times to improve picture quality

To turn processing machine off:-	Turn knob from run to off
	Then close water tap

Switch enlarger and light off

wind enlarger down to lowest position

APPENDIX 3

BLOOD CELL MEASUREMENTS

The technique is easiest when fish are large enough to bleed using a hypodermic syringe and needle. Blood may be taken from the caudal blood vessel which runs ventrally to the spine and can be reached by inserting the needle from the ventral or lateral surfaces of the caudal peduncle.

A) Remove a small blood sample and place 1 drop on a glass slide. Smear the drop along the slide using the edge of another slide. Leave to dry. If the slides are to be stored before staining, fix by dipping in methanol.

B) Stain the slides in Wright's blood stain for 2 minutes. Transfer to a 1 : 1 mixture of Wright's blood stain : Sorensen's buffer for 3 minutes, then rinse in distilled water. Leave to dry.

Wright's blood stain; dissolve 0.3 g solid Wright's stain in 100 ml methanol and 3 ml glyerol. Prepare at least 24 hours before use. Test by placing one drop of stain on a piece of filter paper; if the drop spreads out to leave an even blue/purple stain, the stain is good. If there is a pink halo, do not use.

Sorensen's buffer; dissolve 9.47 g of Na_2HPO_4 in 1 l of distilled water, adjust to pH 6.7

C) Mount using cover slips and DPX mountant or similar.

Each slide should contain areas where the red blood cells are not overlapping and are evenly stained. Using a graticule, measure the major (length) and minor (width) axes of the nuclei of at least 20 cells per slide (these can come from 2 or 3 different areas of the slide to check for consistency). Mean nucleus major axis, minor axis and volume can be calculated for each cell and frequency distributions constructed for control and experimental groups. This should be done in conjunction with chromosome preparations and the accuracy of each nuclear parameter as a ploidy determination tool determined. Nucleus major axis and volume are generally much more accurate than minor axis.

Nucleus volume = $4/3\ ab^2$ where a = major axis/2; b = minor axis/2

APPENDIX 4

PROTOCOL FOR TISSUE HOMOGENIZING FOR ISOZYME

Homogenizing buffer prior to electrophoresis:

10 mM Tris HCl pH 7.1-7.5

1 mM EDTA

1 mM Mercaptoethanol

For AAT (Aspartate aminotransferase); add Pyridoxal-5-phosphate (P5P) 10-25 mg /100 ml of buffer

For IDHP (Isocitrate dehydrogenase) and G6PDH (Glucose-6-phosphate dehydrogenase); add Nicotinamide adenine dinucleotide phosphate (NADP) 10 mg / 100 ml of buffer

Add 1-4 volumes of homogenizing buffer into the homogenized tissue (Sample tissue can be stored at this point as covered tissues, which give better activity).

Homogenize for no longer than 30 seconds, rechill every 8-10 seconds for grinding. Centrifuge at 13,000 g for 15 minutes at 0-4° C.

APPENDIX 5

ELECTROPHORETIC BUFFER AND STAINING RECIPES

A) Buffer solutions used in electrophoresis

TRIS CITRATE pH 8 (TC)

Stock solution :

(0.250 M) Tris	30.29 g/l
(0.075M) Citric acid	15.76 g/l

Adjust to the desired pH 8 before making up volume

Electrode: Undiluted stock solution

Gel: 1: 25 dilution of stock solution

TRIS-BORATE-EDTA pH 8.5 (TBE)

Stock solution :

(0.500 M) Tris	60.57 g/l
(0.240 M) Boric acid	15.00 g/l
(0.016 M) EDTA	5.99 g/l

Adjust to the desired pH 8.5 before making up volume

Electrode: Undiluted stock solution

Gel : 1: 10 dilution of stock solution

TRIS-CITRATE / LITHIUM-BORATE pH 8.5 (TCB)

Stock solution A :

(0.300 M) Boric acid	18.55 g/l
0.100 M) Lithium hydroxide	8.40 g/l

Stock solution B:

(0.076 M) Tris	9.21 g/l
(0.005 M) Citric acid	1.05 g/l
(0.015 M) Boric acid	0.93 g/l
(0.005 M) lithium hydroxide	0.21 g/l

Adjust to desired pH 8.5 before making up volume

Electrode: undiluted stock solution A

Gel: undiluted stock solution B

TRIS-HCl staining buffer series

0.2 M Tris-HCl pH 8 : dilute Tris 24.2 g in 1 l of distilled water, adjust pH to 8 with 5 M HCl

0.1 M Tris-HCl pH 7.4 : dilute Tris 12.1 g in 1 l of distilled water, ajust pH to 7.4 with 5 M HCl

B) The staining recipes for various enzymes

AAT (Aspartate aminotransferase, EC 2.6.1.1) (Dimer)

Tris	300 mg
L-Aspartic acid	65 mg
α-Ketoglutaric acid	20 mg
Pyridoxal-5-phosphate (P5P)	10 mg
Polyvinylpyrrlidone (PVP)	10 mg
Fast blue RR salt	25 mg

Dissolve in 25 ml distrilled water, and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 20-30 mins.

ADA (Adenosine deaminase, EC 3.5.4.4) (Monomer)

Adenosine	15 mg
MTT (Methyl thiazolyl blue)	5 mg
PMS (Phenazine methosulphate)	1 mg
Xanthine oxidase	0.025 units
Nucleoside phosphorylase	0.625 units

Dissolve in 25 ml 0.05 M phosphate buffer pH 7.8, and then add 25 ml 2 % agar. Incybate the gel slice at 37° C for 15-20 mins.

FH (Fumarate hydratase, EC 4.2.1.2) (Tetramer)

Sodium fumarate (Fumaric acid)	60 mg
NAD (Nicotinamide adenine dinucleotide)	20 mg
Sodium pyruvate (Pyruvic acid)	(20 µl) 20 mg
Malic dehydrogenase	60 units
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.5 M tris-HCl pH 8 and then add 20 ml 2 % agar. Incubate the gel slice at 37° C for 15-20 mins.

G3PDH (Glycerol-3- phosphate dehydrogenase, EC 1.1.1.8) (Dimer)

DL-α-glycerophosphate	200 mg
Sodium pyruvate (Pyruvic acid)	200 mg
EDTA	60 mg
NAD	15 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 20 ml 2 % agar. Incubate the gel slice at 37° C for 10-15 mins.

G6PDH (Glucose-6-phosphate dehydrogenase, EC 1.1.1.49) (Dimer)

D-Glucose-6-phosphate	10 mg
NADP (Nicotinamide adenine dinucleotide phosphate)	5 mg
1 M $MgCl_2$	1 ml
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gelslice at 37° C for 20-30 mins.

GPI (Glucose-6-phsphate isomerase, EC 5.3.1.9) (Dimer)

D- Fructose-6-phosphate	20 mg
NADP	5 mg
1 M $MgCL_2$	100 ml
MTT	5 mg
PMS	1 mg
Glucose-6-phosphate dehydrogenase	1.4 units

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 7-15 mins.

IDPH (Isocitrate dehydrogenase NADP+, EC 1.1.1.42) (Dimer)

DL-Isocitric acid	50 mg
NADP	6 mg
MTT	7 mg
PMS	1 mg
1 M $MgCl_2$	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 15-20 mins.

LDH (L-Lactate dehydrogenase, EC 1.1.1.27) (Tetramer)

Sodium lactate (solution)	200 µl
NAD	10 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 10-15 mins.

MDH (Malate dehydrogenase, EC 1.1.1.37) (Dimer)

DL-Malic acid	60 mg
NAD	10 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 10-15 mins.

PGDH (Phosphogluconate dehydrogenase, EC 1.1.1.44) (Dimer)

6-Phosphogluconate (Na3) (6-phosphogluconic acid)	10 mg
1 M $MgCl_2$	100 µl
NADP	5 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 10-15 mins.

PGM (Phosphoglucomutase, EC 5.4.2.2) (Monomer)

α-D-Glucose-1-phosphate (Sodium)	50 mg
1 M $MgCl_2$	350 µl
NADP	5 mg

MTT	5 mg
PMS	1 mg
Glucose-6-phosphate dehydrogenase	1.4 units

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 5-10 mins.

SOD (Superoxide dismutase, EC 1.15.1.1) (Dimer)

NAD	10 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C or exposed to light at ambient temperature.

XDH (Xanthine dehydrogenase, EC 1.2.1.37 or 1.1.1.204) (Monomer/Dimer)

Hypoxanthine	20 mg
NAD	15 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 15-20 mins.

XOD (Xanthine oxide dismutase, EC 1.1.3.22) (Dimer)

Hypoxanthine	15 mg
MTT	5 mg
PMS	1 mg

Dissolve in 25 ml 0.2 M Tris-HCl pH 8 and then add 25 ml 2 % agar. Incubate the gel slice at 37° C for 15-20 mins.

C) Fixing solution for starch gel stain

Glacial acetic acid	200 ml
Methanol, (ethanol)	1,000 ml
Distilled water	1,000 ml

Mix thouroughly

D) Calculating formulas employed

1. Chi-square test for goodness-of-fit (Sokal& Rohlf, 1969)

$$\chi^2 = \Sigma\ (\text{Obs-Exp})^2(\text{Exp})^{-1}$$

'Obs' = observed genotype frequencies

'Exp' = expected genotype frequencies

The expected genotype frequencies were calculated using Levene's (1949) formula for small samples.

$$E(X_{ii}) = Y_i\,(Y_i\text{-}1)(4n\text{-}2)^{-1}$$

$$E(X_{ij}) = Y_i\,Y_j(2n\text{-}1)^{-1}$$

Where $X_i i$ = the number of a_i/a_i homozygotes in the sample, X_{ij} = the number of a_i/a_j heterozygotes in the sample, Y_i = the number of ai alleles in the sample, Y_j = the number of a_j alleles in the sample, and n = the sample size.

2 Expected heterozygosity (unbiased estimate of Nei, 1978)

For a single locus, an unbiased estimate of heterozygosity is given by

$$h = 2n(1\text{-}\Sigma p_i^2)/(2n\text{-}1),$$

whereas the corresponding unbiased estimat of H averaged over all loci is

$$H = \Sigma h_k/r\ ,$$

where p_i is the frequency of the ith allele at a locus in a sample from the population, h_k the value of h for the kth locus, and r the total number of loci investigated. The sample size n may be vary from locus to locus.

$$S.E. = \sqrt{Var}$$

$$Var = \Sigma(h_k\text{-}H)/r(r\text{-}1)$$

APPENDIX 6

HISTOLOGICAL PROCEDURE

A) Fixation

The aim of fixation is :

1. To prevent autolysis, bacterial decomposition and putrefaction
2. To coagulate the tissue so to prevent loss of easily diffusible substances such as glycogen.
3. To safeguard the tissue against the damaging effects of tissue processing.
4. To leave tissues in a condition which facilitates differential staining with dyes and other reagents.

Material for histological examination should be placed in fixative for at least 24 hours prior to cassetting. 10 % neutral buffered formal saline is normally used. One volume of tissue per 25 volumes of fixative is recommended. Individual tissues must be of a suitable size to allow permeability of fixative.

Formalin is an irritant of eyes, nose and skin. It should be handled only in a fume cabinet and disposable gloves worn whilst handling and dissecting sample.

10 % Neutral Buffer Formalin

Sodium dihydrogen phosphate (monohydrate)	4.0 g
Sodium hydrogen phosphate (anhydrous)	6.5 g
Formaldehyde	100 ml
Distilled water	900 ml

Bouin's fixative Solution

Glacial acetic acid	1 part
Formalin (37% formadehyde)	5 parts
Saturated aqueous picric acid	15 parts

Other commonly used fixatives include Gluteraldehyde, Carnoy's and Davidson's.

B) Cassetting

The allocated case number is entered on the cassette using a pencil (ink will be removed by solvents during processing).

Tissue samples should be trimmed to a suitable size and must not be overcrowded in cassettes as this will lead to ineffective dehydration and ultimately difficulty in sectioning.

Small samples are wrapped in tissue paper before placing in the cassette. Soft and hard tissues should be kept separate. Cassetted samples should not be allowed to dry out and must be left in a bowl of water or fixative until loading onto the processor.

C) Processing

The aim of processing is to impregnate the tissue with an embedding medium which will give support to the tissue during section cutting. Paraffin wax embedding is most commonly used.

First, water is removed from the tissues by immersing them in a graded series of alcohols, ending in absolute alcohol. This is followed by immersion in a clearing

agent (Chloroform) which is miscible with both alcohol and wax. The clearing agent is therefore easily removed by the molten wax in the final stage.

This procedure is carried out by placing the cassettes into a basket which is moved round automatically by a tissue processor at the appropriate time intervals.

Schedule :-

1. 50 % Methylated Spirit 1 hr.
2. 80 % Methylated Spirit 2 hrs.
3. 100 % Methylated Spirit 2 hrs.
4. 100 % Methylated Spirit 2 hrs.
5. 100 % Methylated Spirit 2 hrs.
6. 100 % Alcohol 2 hrs.
7. 100 % Alcohol 2 hrs.
8. Chloroform 2 hrs.
9. Chloroform 1 hr.
10. Molten Wax 1 hr.
11. Molten Wax 2 hrs.
12. Molten Wax 2 hrs.

D) Blocking out

Cassettes are removed from the processor and placed in molten wax until ready to block out.

The metal lid is removed and the appropriate size of base mould selected to give an adequate margin of wax around the tissue. The base is filled with wax and the

tissue sample pressed into the wax. The empty cassette is placed on top of the mould and topped up with wax.

By placing the mould on the cold plate the wax solidifies and the sample is held in position. Orientation of the tissue depends primarily on the type of section required. Tissues such as skin should be embedded so that the skin edge is uppermost in the block. This makes sectioning easier as the soft tissue underneath is cut through before the hard skin surface. The wax is allowed to solidify on a cold plate before removing to cut sections.

E) Microtomy

1. Trimming in

The surface layer of wax has to first be removed to expose the complete surface of the specimen. This is carried out on the microtome using an old blade. The rate of advancement of the block towards the knife is determined manually at this stage.

2. Section cutting

Before sections can be obtained from blocks of hard tissue it is often necessary to surface decalcify them. This is carried out by placing blocks face down in a vessel containing a layer of decalcifying solution for approximately 1 hour. Blocks of soft tissue which have been hardened excessively during processing can be soaked in water.

Blocks are cooled using a cold plate prior to sectioning.

Specimens are clamped into the block holder which is automatically advanced every rotation of the operating wheel (normally 5 μm). When a "ribbon" is obtained this is removed and floated out on a water bath. The best section is selected and picked up on a clean glass slide. The case number is marked on the slide using a diamond pencil and the slide is placed face down on a hotplate. Racked slides are then dried in an oven at 60° C for at least one hour before staining.

F) Staining

In order to examine sections effectively under the microscope they require to be stained. Many different staining techniques can be used, the most common being Haematoxylin and Eosin.

1. Xylene	5 min.
2. Alcohol I	2 min.
3. Methylated Spirits	1.5 min.
4. Running tap water	wash
5. Haematoxylin	5 min.
6. Wash in tap water	
7. Acid Alcohol	3 quick dip.
8. Wash in tap water	
9. Scott's tap water	30 sec.
Check staining microscopically at this stage	
10. Wash well	
11. Eosin	5 min.
12. Quick wash in tap water	
13. Methylated spirit	30 sec.
14. Alcohol II	2 min.
15. Alcohol I	1.5 min.

16. Xylene	5 min.
17. Xylene	

Sections must not be left out of Xylene as they will dehydrate. Sections are coverslipped after the last Xylene in the staining series. Once coverslipped, sections are labelled with the case number, and mounting fluid allowed to dry before examination.