Mass Production of Beneficial Organisms

Invertebrates and Entomopathogens

Edited by
Juan A. Morales-Ramos, M. Guadalupe Rojas, and David I. Shapiro-Ilan

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Edited by

Juan A. Morales-Ramos
Biological Control of Pest Research Unit, National Biological Control Laboratory,
USDA-Agricultural Research Service, Stoneville, MS, United States

M. Guadalupe Rojas
Biological Control of Pest Research Unit, National Biological Control Laboratory,
USDA-Agricultural Research Service, Stoneville, MS, United States

David I. Shapiro-Ilan
USDA-ARS, Southeastern, Fruit and Tree Nut Research Unit, Byron, GA, United States

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Dedication

We dedicate this book to W. Louis Tedders (1953–2013). Louis was a Research Entomologist with the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) for almost 35 years. Subsequently, he was the CEO of Southeastern Insectaries, Inc. The company focused on the production of mealworms and entomopathogenic nematodes. Louis had an immense impact on the field of biological control and mass production of beneficial organisms. He worked extensively with various biocontrol organisms, including arthropod predators and parasitoids as well as insect pathogens. Based on his exceptional ingenuity and imagination, Louis was an inventor on eight patents, and the technology he developed has been adopted widely across various commodities around the globe. Louis had an infectious level of excitement and curiosity for entomological research; he loved the field! Louis was a beloved mentor to the editors of this volume; we miss him greatly!

Juan A. Morales-Ramos
M. Guadalupe Rojas
David I. Shapiro-Ilan

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Contents

List of contributors

Hugo Arredondo-Bernal Centro Nacional de Referencia de Control Biológico (National Center of Biological Control Reference), Dirección General de Sanidad Vegetal (Directorate General of Plant Helth), Tecomán, Colima, Mexico

Derek R. Artz US Department of Agriculture, Agricultural Research Service (USDA-ARS), Pollinating Insects Research Unit, Logan, UT, United States

Robert Behle USDA-ARS-NCAUR, Crop Bioprotection Research Unit, Peoria, IL, United States

Ilaria Biasato Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy

Tim Birthisel The Andersons Inc., Turf and Specialties Group (retired), Asheville, NC, United States

Karel Bolckmans Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Kevin R. Butt Earthworm Research Group, University of Central Lancashire, Preston, United Kingdom

Leslie Chan Thermo Fisher Scientific, Brisbane, QLD, Australia

Matthew A. Ciomperlik USDA-APHIS-PPQ, Edinburg, TX, United States

Terry L. Couch Becker Microbial Products Inc., Parkland, FL, United States

Thomas A. Coudron Biological Control of Insects Research Laboratory, USDA-Agricultural Research Service, Columbia, MO, United States

Rosemarie De Clerck-Floate Agriculture and Agri-Food Canada, Lethbridge Research & Development Centre, Lethbridge, AB, Canada

Patrick De Clercq Department of Plants and Crops, Ghent University, Ghent, Belgium

Henry de Malmanche School of Chemistry & Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia

Maria Luisa Dindo Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy

Marcus V.A. Duarte Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Paula Enes CIIMAR—Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Matosinhos, Portugal; Department of Biology, Faculty of Sciences of University of Porto, Porto, Portugal

Mark D. Finke Mark Finke LLC, Rio Verde, AZ, United States; Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, the Netherlands

Tarra A. Freel EnviroFlight, LLC, Maysville, KY, United States

Francesco Gai Institute of Sciences of Food Production, National Research Council, Grugliasco, Italy

M.D. García-Cancino Laboratorio Regional de Reproducción de Tamarixia radiata (Regional Laboratory for reproduction of Tamarixia radiata), Centro Nacional de Referencia de Control Biológico-CNRF, Mérida, Yucatán, Mexico

Laura Gasco Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy

Chris Geden USDA-ARA, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL, United States

John A. Goolsby USDA-ARS, Knipling-Bushland Livestock Insects Research Laboratory, Cattle Fever Tick Research Unit, Edinburg, TX, United States

Juli R. Gould USDA-APHIS-PPQ, Otis AFB, MA, United States

Simon Grenier Rue des Mésanges, Chassieu, France

David Grzywacz Agriculture, Health and Environment Department, University of Greenwich, Central Avenue, Chatham Maritime, Kent, United Kingdom

Mallory A. Hagadorn Department of Biology, Utah State University, Logan, UT, United States

Richou Han Guangdong Entomological Institute, Guangzhou, P.R. China

Martin P. Hill Centre for Biological Control, Department of Zoology and Entomology, Rhodes University, Makhanda (Grahamstown), EC, South Africa

Kim A. Hoelmer USDA-ARS Beneficial Insects Introduction Research Unit, Newark, DE, United States

Man P. Huynh Division of Plant Science & Technology, University of Missouri, Columbia, MO, United States; Department of Plant Protection, Can Tho University, Can Tho, Vietnam

Trevor A. Jackson AgResearch, Lincoln Research Centre, Private Bag 4749, Canterbury Region, Christchurch, New Zealand

Stefan T. Jaronski Jaronski Mycological Consulting LLC, Blacksburg, VA, United States

Juan Luis Jurat-Fuentes Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN, United States

Elizabeth A. Koutsos EnviroFlight, LLC, Maysville, KY, United States

Luis Garrigós Leite Institute of Biology, APTA, São Paulo, SP, Brazil

Norman C. Leppla Entomology and Nematology Department, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, FL, United States

Thuy-Tien T. Lindsay USDA-ARS Pollinating Insects Biology, Management and Systematics Research Unit, Logan, UT, United States

Kimberly A. Livingston College of Veterinary Medicine, North Carolina State University, Raleigh, NC, United States

Christopher N. Lowe Earthworm Research Group, University of Central Lancashire, Preston, United Kingdom

Belinda Luke CABI, Bakeham Lane, Egham, Surrey, United Kingdom

Rosemary Malfi Department of Biology, University of Massachusetts, Amherst, MA, United States

David Moore CABI, Bakeham Lane, Egham, Surrey, United Kingdom

Sean Moore Citrus Research International, Walmer, Port Elizabeth, South Africa

Juan A. Morales-Ramos Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States

Patrick J. Moran U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Invasive Species and Pollinator Health Research Unit, Albany, CA, United States

Dennis Oonincx Mark Finke LLC, Rio Verde, AZ, United States; Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, the Netherlands

Paul H. Patterson Department of Animal Science, The Pennsylvania State University, University Park, PA, United States

Quentin Paynter Manaaki Whenua - Landcare Research, Biocontrol and Molecular Ecology Team, Auckland, New Zealand

Apostolos Pekas Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Stephen S. Peterson AgPollen LLC, Visalia, CA, United States

Holly Popham AgBiTech, Columbia, MO, United States

R.J. Rabindra Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

S. Raghu Commonwealth Science Industry and Research Organisation (CSIRO), Brisbane, QLD, Australia

Steven Reid School of Chemistry & Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia

Eric W. Riddick Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States

B. Rodríguez-Vélez Centro Nacional de Referencia de Control Biológico (National Center of Biological Control Reference), Dirección General de Sanidad Vegetal (Directorate General of Plant Helth), Tecomán, Colima, Mexico

M. Guadalupe Rojas Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States

Genevieve Rowe Wildlife Preservation Canada, Guelph, ON, Canada

David I. Shapiro-Ilan USDA-ARS, Southeastern Fruit and Tree Nut Research Unit, Byron, GA, United States

Kent S. Shelby Biological Control of Insects Research Laboratory, USDA-Agricultural Research Service, Columbia, MO, United States

Rhonda L. Sherman Department of Horticulture Science, North Carolina State University, Raleigh, NC, United States

Gregory S. Simmons USDA-APHIS-PPQ, Salinas, CA, United States

James P. Strange Department of Entomology, The Ohio State University, Columbus, OH, United States

Sevgan Subramanian Environmental Health Theme, International Centre of Insect Physiology and Ecology, Nairobi, Kenya

Monique M. van Oers Laboratory of Virology, Wageningen University and Research, Wageningen, the Netherlands

Dominiek Vangansbeke Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Felix Wäckers Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Neal M. Williams Department of Entomology and Nematology, University of California, Davis, CA, United States

Deyu Zou Biological Control of Insect Research Laboratory, Institute of Plant Protection, Tianjin Academy of Agricultural Sciences, Tianjin, P.R. China

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Preface

Novel technology is needed to secure a sustainable future in agriculture and other fields associated with the production of food. Current food production practices rely on the use of chemical pesticides and synthetic fertilizers, which produce continuous and progressive environmental deterioration. Livestock production requires large areas of land and large quantities of water but produces large quantities of waste. Current formulations of feed for aquaculture and poultry rely on the use of fish meal to provide essential amino acids and vitamins lacking or in low supply in products of vegetable origin. Fish meal is a byproduct of fisheries, which are becoming increasingly unsustainable worldwide. Since 1991 aquaculture contributions to total fish production has been increasing worldwide and had reached 45% in 2016 (FAO, 2018). The production of some invertebrates and certain microbes can contribute to solving these problems by providing biological control agents to reduce the use of pesticides, providing pollinators, improving soil health, and producing alternative sources of animal protein for feed formulations that are more sustainable.

Mass Production of Beneficial Organisms, in its second edition, includes new chapters dealing with production of mite predators, use of insects as food for aquaculture and poultry and expands chapters to cover production of hymenopteran parasitoids and biocontrol agents for weeds. In addition, almost every chapter has been updated with new information. This book will focus on methods of producing beneficial insects, mites, heartworms, entomopathogenic viruses, bacteria, fungi, and nematodes and provide examples of their use and potential applications for the future. Chapters are grouped in three sections: Section I includes production methods for insects and mites used as biological control agents and methods for quality control and the development of artificial diets. This section is a comprehensive review of the application of insect and mite rearing to the biological control of pests and all past experiences that have led to important successes in the augmentation of natural enemies to control important pests. Although the use of parasitoids and predators in biological control has been more successful in enclosed agriculture such as greenhouses and high tunnels, its past successes hint toward a great potential for the future as the technology for insect mass production improves.

Section II includes methods of production of entomopathogens and methods for their formulation. The most commercially successful form of biological control has been the use of entomopathogens, which can be produced, formulated, and applied more consistently. Microbial control as a branch of biological control continues to be an integral part of current integrated pest management practices. Entomopathogens hold the promise to be so successful as to be capable of replacing chemical pesticides.

Section III includes perspectives and methods for producing insects and earthworms for diverse uses including as animal feed, human food, and for pollination. In recent years the FAO has recognized the potential of insects as a source of food and animal feed, which could lead to a more sustainable future. Insects have been part of the human diet since ancient times and some studies suggest that a diet including insects can be healthier. Production of insects is considered more sustainable than the production of other animal sources of food because it requires less space, produces less or no greenhouse gasses, less water and energy, and insects are more efficient food converters. However, the most immediate and important contribution of insect production could be to provide an alternative to fish meal for the formulation of feeds for aquaculture, poultry, and other livestock production. Production of pollinators is another important area, which could contribute to reduce current problems associated with the phenomenon of colony collapse syndrome in honeybees. One of the most compelling theories on the origin of this syndrome is the current overuse and over transportation of honeybee colonies to satisfy pollination demands in vast agricultural areas. Production of alternative species of pollinators could help to alleviate this by providing local sources of pollinators.

Production of beneficial organisms presents a source of new directions for agriculture and industry into more sustainable and environmentally friendly methods of producing food. We hope that the information presented in this book will stimulate a new generation of scientist and entrepreneurs in growing this important branch of agricultural science.

Reference

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Section I

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Chapter 1

Introduction

Norman C. Leppla[1], Juan A. Morales-Ramos[2], David I. Shapiro-Ilan[3] and M. Guadalupe Rojas[2]

[1]*Entomology and Nematology Department, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, FL, United States,* [2]*Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States,* [3]*USDA-ARS, Southeastern Fruit and Tree Nut Research Unit, Byron, GA, United States*

1.1 Challenges of mass-producing beneficial organisms

Mass Production of Beneficial Organisms contains chapters on producing selected organisms useful to humankind, including arthropods, microorganisms, bees, and earthworms. It is comprised of a series of comprehensive descriptions of the industrial-level production of insects, mites, and pathogens for biological control, and beneficial invertebrate organisms for food, feed, pollination, and other purposes. Additionally, there are reports on artificial diet development and quality assurance for arthropods, as well as entomopathogen production and formulation. The final section covers insects as food for domestic animals, insectivores, and humans, along with solitary bees for pollination and earthworm mass culture. This is a unique assemblage of topics organized around the goal of producing large amounts of organisms for a variety of useful purposes.

Mass production of these organisms is somewhat arbitrary to define in terms of the number produced per time interval. Rather, it is characterized by the magnitude and degree of separation of the rearing processes, usually involving a single species. It takes place in large, multiroom mass-rearing facilities or "biofactories" specially designed for this purpose. There is a trained labor force with at least one employee assigned to each independent rearing process, such as diet preparation or another single production activity. Depending on the species being produced, large amounts of the host material, artificial diet ingredients, or growth media are used and there usually is some mechanization to make the rearing more efficient. Thus, mass production of beneficial organisms can be considered an industrial process with all of the associated logistical requirements, including substantial quantities of production materials, continuous maintenance of facilities and equipment, and distribution of high-quality products (see Chapter 9).

Principles and procedures for mass-producing beneficial arthropods and microbes have developed independently, although there are commonalities. Both kinds of organisms are produced in biological systems that depend on genetically suitable founding populations, uncontaminated diets or media, mechanized equipment, controlled environments, quality assurance, packaging, and delivery to customers as effective products. The subjects encompassed in principles and procedures for developing and operating production systems for these organisms can be divided into the following: facility design and management, including health and safety; environmental biology; management of microbial contamination; nutrition and diet; population genetics; and quality control (Schneider, 2009). Unlike general principles, however, procedures are typically species-specific in terms of diet or substrate and associated culturing methods. A suitable host organism must be used in the absence of an artificial diet to rear a parasitic or predatory arthropod and, similarly, beneficial microorganisms often are cultured on a defined artificial medium or, when in vitro culture is not feasible, on susceptible hosts. Regardless of species, procedures for mass rearing any beneficial organism are divided into a series of steps based on its life cycle.

Insect mass production progressed naturally from relatively small-scale rearing of insects for human and animal food, such as honey or mealworms, *Tenebrio molitor* L., or for their products that historically have included silk, cochineal dye, lac, and beeswax. Reliable supplies of insects that behaved normally also were needed for research and teaching (Needham et al., 1937). Blowflies, several species of filth flies, mosquitoes, and the common bed bug, *Cimex lectularius* L., have been essential for the advancement of medical and veterinary research. These insects and vectors of human and animal pathogens, such as mosquitoes and the tsetse fly, *Glossina* spp., were used to screen chemical compounds for

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efficacy and toxicity. *Drosophila melanogaster* Meigen became the standard insect model for genetic research. For crop protection, large numbers of several insect species were needed for studies on host plant resistance to insects, including certain pests Heteroptera, Diptera, Coleoptera, and Lepidoptera, such as the European corn borer, *Ostrinia nubilalis* (Hubner). Commodity treatments were developed for the Khapra beetle, *Trogoderma granarium* Everts, additional species of grain-infesting beetles and moths, several kinds of tephritid fruit flies, and many other types of insects. Large quantities of the boll weevil, *Anthonomus grandis* Boheman, noctuid moths, the pink bollworm, *Pectinophora gossypiella* (Saunders), and other Coleoptera and Lepidoptera were used to develop attractants and traps. Some of these insects also provided hosts for rearing imported natural enemies in quarantine prior to release in the field. Moreover, most predators and parasitoids used in augmentative biological control require massive amounts of natural and factitious hosts. These hosts typically are more difficult to rear consistently than the natural enemy itself. Due to this host rearing limitation, large quantities of insects for release in autocidal control must be produced on artificial diets.

More than 50 species of arthropod natural enemies are produced in large enough numbers to be marketed widely in the United States (LeBeck and Leppla, 2021) and almost 350 species are potentially available globally (van Lenteren, 2003; van Lenteren et al., 2018). Popular predators include several phytoseiid mites, coccinellids, cecidomyiids, and chrysopids, and the most commonly used parasitic wasps are in the taxonomic families Aphelinidae, Braconidae, Pteromalidae, and Trichogrammatidae. Predaceous mites are applied extensively for biological control of phytophagous mites, fungus gnats, and thrips on potted and bedding plants in protected cultures and interiorscapes. They are particularly useful for two-spotted spider mite, *Tetranychus urticae* (Koch), control on ornamental, fruit, and vegetable crops. Depending on the species, lady beetles are released to control a variety of scales, mealybugs, aphids, thrips, and whiteflies. The cecidomyiid, *Aphidoletes aphidimyza* (Rondani), is often used for aphid biological control, as are *Chrysoperla* spp. Lacewings. and *Aphidius* spp. Probably the most popular aphelinid is *Encarsia formosa* Gahan, released extensively in greenhouses to control whiteflies. A specialized purpose for mass-reared natural enemies is the use of pteromalids for biological control of filth flies in manure and compost. There are ample opportunities to develop and implement augmentative biological control in the United States and globally for agriculture, forestry, rangeland, protected culture, and other environments (van Lenteren, 2012; Barratt et al., 2018; van Lenteren et al., 2018; Wyckhuys et al., 2018).

Numerous species of the lepidopteran egg parasitoid, *Trichogramma* spp., have been mass-produced on factitious hosts in semimechanized rearing facilities for decades, becoming the most prevalent augmentative parasitoid in both number of production facilities and quantities produced. They typically are reared on eggs of the angoumois grain moth, *Sitotroga cerealella* (Oliver), or Mediterranean flour moth, *Ephestia kuehniella* Zeller, that infest stored grain on which they are reared (Moghaddassi et al., 2019). Biofactories in Europe and Asia have consistently produced millions of *Trichogramma* spp. per day for years to control the pest Lepidoptera in field crops (Hassan, 1993; Smith, 1996; Stefanovska et al., 2006). A highly successful European corn borer biological control project has been conducted in Germany, Switzerland, and France since about 1992 (Kabiri and Bigler, 1996). Producers of commercial natural enemies and collaborative government/grower groups throughout the world have developed a variety of simple, highly productive rearing systems for *Trichogramma* spp. In every situation, large containers of grain are infested with host eggs, yielding larvae that feed and eventually molt into adults that deposit eggs. The eggs are harvested, exposed to *Trichogramma* spp. adults, and used to maintain the colony or attached to a substrate for distribution in a crop. Periodically, eggs of the target pest are substituted for the factitious host to maintain high levels of pest parasitism.

Arthropod mass rearing reached an industrial level with the development of autocidal control and eradication of the New World screwworm fly, *Cochliomyia hominivorax* (Coquerel). The first large-scale production facility was established near Sebring, Florida in a converted surplus US Air Force hangar (Baumhover, 2002). During the early period of screwworm eradication in the southeastern United States, 50 million flies were produced weekly from larvae reared on meat of cattle, horses, pigs, whales, and nutria, *Myocaster coypus* (Molina). Production was moved to Mission, Texas and increased to 75−200 million per week to support eradication of the fly from the southwestern United States. As eradication progressed further south, the biofactory in Texas was closed and a new one established at Tuxtla Gutierrez, Mexico to produce 250−300 million flies per week (Meyer, 1987). Rearing each screwworm generation began when female flies oviposited egg masses on wooden frames treated with spent larval medium. The eggs were scraped from the frames, incubated, and placed on small pieces of lean meat before being transferred to a liquid diet as first instar larvae. The larvae developed to maturity on liquid larval diets composed of various formulations of lean ground beef, citrated beef blood, powdered milk, water, and formalin dispensed onto cotton linters during the early years and subsequently cellulose acetate blankets in shallow trays. The final and most complex liquid larval diet contained dried whole chicken egg, dried whole bovine blood, powdered milk substitute, sucrose, dried cottage cheese, and formalin (Taylor, 1992; Chen et al., 2014). Mature larvae left the trays, fell into a water stream, and were collected for pupation, sterilization, and release from airplanes as adults. Larval rearing became more efficient by incorporating the liquid diet ingredients into a gelling agent and eliminating the acetate

mats. However, the flies continued to be fed ground beef mixed with honey. Because mass production of the screwworm enabled this pest to be eradicated from virtually all of North America, except some Caribbean islands, the rearing facility was relocated to Pacora, Panama (Scott et al., 2017). There, state-of-the-art insect mass-rearing facilities, equipment, materials and methods have been established and continuously improved.

Another pioneering insect mass rearing capability was developed for the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), a global pest of tropical fruit and citrus. In North America, the sterile insect technique was developed for medfly by adapting concepts and methods proven successful for eradicating the screwworm. A biofactory was built at Metapa, Mexico and a mass-rearing system was developed based primarily on research conducted at Seibersdorf, Austria, Costa Rica, Hawaii, and a few other locations. The flies were held in large cages and fed granulated sugar and protein hydrolysate formed into dry cakes. Water was provided in tubes fitted with absorbent wicks. The flies oviposited on nylon cloth sheets from which the eggs were washed into a water bath for collection and incubation. The initial larval diet was a suspension of soy flour, wheat bran, granulated sugar, torula yeast, methyl parahydroxybenzoate, and water mixed into sugarbeet, *Beta vulgaris* L., bagasse. The bagasse often was of poor quality and sources became unreliable, so it was replaced with a variety of starch materials, such as corncob grits. As with the screwworm, mature medfly larvae leave the diet naturally but, for mass rearing, the medium containing medfly larvae was transferred to large, cylindrical rotating larval separation machines. The larvae then were gathered, placed into pupation trays, and held for adult emergence. The goal was achieved by producing 500 million pupae per week for distribution by air as flies. The medfly was eradicated from the United States and Mexico, except for periodic incursions. Eventually, at least 14 medfly mass-rearing facilities were built throughout the world, the largest at El Pino, Guatemala with a maximum production of 2 billion plus per week (Tween, 2002).

Recently, there has been considerable interest in mass-producing insects for use in animal feed and human food. Insects, such as the house cricket, *Acheta domesticus* L., and the yellow mealworm have been produced commercially for the past 60 years for pet food and fish bait in the United States (Cortes Ortiz et al., 2016). However, early producers have developed rearing techniques with limited support from scientific research due to the lack of funding for projects associated with the use of insects as food. This changed after release of an FAO report on the potential of insects as an alternative source of feed and food (van Huis et al., 2014). Since this report was released, publication of articles on the use of insects as animal feed and for human consumption (entomophagy) has grown exponentially. Mass production of the black soldier fly, *Hermetia illucens* L., for animal feed has been studied extensively and resulted in the creation of a new industry. Great emphasis also has been placed on the potential of insects as ingredients in feed formulations for aquaculture and poultry using different species, including the yellow mealworm, black soldier fly, house fly, *Musca domestica* L.*;* and silkworm, *Bombyx mori* (L.) (Bondari and Sheppard, 1987; Ng et al., 2001; Fasakin et al., 2003; Barroso et al., 2014; Makkar et al., 2014; De Marco et al., 2015; Sánchez-Muros et al., 2016; Gasco et al., 2016, 2018, 2019; Lock et al., 2018; Ferrer Llegostera et al., 2019; Benzertiha et al., 2019). Current feed formulations for cultured fish and poultry rely on a fish meal to supply essential amino acids, minerals and select vitamins for adequate nutrition. Because fish meal production requires unsustainable harvesting of oceanic fish, the animal production industry is looking for a more sustainable alternative to supplement animal feed formulations (Food and Agriculture Organization of the United Nations, 2018). Mass-reared insects are becoming increasingly important for this purpose as shortages of animal and plant protein become more frequent (Govorushko, 2019; Sogari et al., 2019; Rumbos and Athanassiou, 2021). Applications of commercially produced insects for feed are discussed in Chapters 16 and 17.

1.2 Challenges of arthropod mass production for biological control

Mass production and release of arthropod natural enemies is the foundation of augmentative biological control (King, 1993; Elzen and King, 1999; Morales-Ramos and Rojas, 2003). It is a complex process that often involves a multidisciplinary effort and substantial economic investment to develop the technology, construct adequate facilities, and hire and train personnel. The investment needed to establish mass production systems for new arthropod species can only be met by large government or industry organizations. Effective use of biological control agents to control major pests in crops involves the release of tens of thousands to millions of predators or parasitoids (King et al., 1985). Knowledge has accumulated over decades on small-scale rearing of arthropod natural enemies, but new technologies are needed to increase production capabilities from thousands to millions of organisms per week.

Government-supported arthropod mass production has been successful for autocidal control programs, such as those for the screwworm, pink bollworm, and Mediterranean fruit fly. Autocidal control usually aims to eradicate the target pest and therefore tends to be temporary. Nevertheless, these programs have contributed essential methods and expertise for advancing insect mass production. The resulting technology has been used in government-supported augmentative

biological control of some key pests, such as the European corn borer and other Lepidoptera. Parasitoid and predator production technologies developed in these programs helped the biological control industry slowly emerge during the 1990s. This industry commercializes arthropod natural enemies for augmentative biological control of pests.

The commercialization of arthropods as biological control agents dramatically changed the direction in which mass production technology evolved. In a free-market economy, mass-produced natural enemies must compete with other pest control technologies to become commercially viable and sustainable. Biological control agents must effectively control the target pests and their cost must be competitive (King et al., 1985; Naranjo et al., 2015). The first section of this book contains chapters that describe commercial successes and failures of mass-produced arthropods intended for the biological control of pests. The chapters cover different arthropod groups and technologies for their mass production and explain the difficulties in bringing them to commercial application.

A limited number of arthropod natural enemies can be mass-produced economically using current technology (Mhina et al., 2016). One of the major obstacles to producing natural enemies is the requirement to produce their host or prey. This doubles the costs by producing two species while generating revenues from only one (van Driesche and Bellows, 1996). Further complications arise from the need to also grow the host plant for the production of host herbivores. As a result, with few exceptions, natural enemies are produced on herbivorous species that have been reared on artificial diets. This has limited the range of natural enemy species that are mass-produced. Predatory arthropods capable of developing and reproducing on easy-to-rear factitious prey have been mass-produced more frequently (van Driesche and Bellows, 1996). Therefore, many commercially produced natural enemies are omnivorous predators capable of feeding on plant materials, including the phitoseiid mites, *Neoseiulus californicus* (McGregor), *N. fallacies* (Garman) (Croft et al., 1998), and *Amblyseius swirkii* Athias-Henriot (Messelink et al., 2008); the insidious flower bug *Orius insidiosus* (Say); *Harmonia axyridis* (Pallas); *Coleomegilla maculata* (deGeer); and *Hippodamia convergens* Guérin-Méneville (Lundgren, 2009).

Developing artificial diets for parasitoids and predators can simplify their mass production, making it more cost-effective. However, artificial diets are often inferior to natural prey or hosts as sources of nutrition for entomophagous species (Grenier, 2009). As a result, parasitoids and predators grown on artificial diets can have characteristics that diminish their quality as biological control agents (Grenier and De Clercq, 2003; Riddick, 2009). Directions for future development of artificial diets are presented in Chapter 7 and methods to evaluate their quality are described in Chapters 8 and 9.

1.3 Challenges of mass-producing pathogens for biological control

Microbial control can be defined as the use of pathogens to suppress pests. Thus, microbial control is a branch of the broader discipline of biological control and may be thought of as applied epizootiology (Shapiro-Ilan et al., 2012). Most researchers also include microbial by-products in their definition of microbial control, such as toxins or metabolites. Furthermore, some consider natural suppression of pests without any human intervention to be included in microbial control, but in this volume, we limit microbial control to intentional manipulation of the targeted system. Microbial control agents (e.g., pathogenic viruses, bacteria, fungi, and protists) can be applied to suppress of weeds, plant diseases, or insects (Tebeest, 1996; Montesinos, 2003; Janisiewicz and Korsten, 2002; Vega and Kaya, 2012). This volume includes chapters on the production of microbial control agents for the suppression of insect pests in the second section. Specifically, it covers the mass production of four major groups of entomopathogens: nematodes (Chapter 10), fungi (Chapter 11), bacteria (Chapter 12), and viruses (Chapter 13). Another group of entomopathogens, the protists, is not included in this book because currently there is no commercial production of these agents.

Chemical pesticides can be harmful to humans and the environment, and may cause secondary pest outbreaks and resistance (Debach, 1974). In contrast, microbial control agents (similar to arthropod biocontrol agents) are safe for humans and the environment, and generally have little or no effect on other nontarget organisms; microbial control agents also generally have a substantially reduced risk of inducing resistance (Lacey and Shapiro-Ilan, 2008). Relative to chemical insecticides, however, microbial control agents have certain disadvantages, such as susceptibility to environmental degradation by ultraviolet light. Additionally, the narrow host range of certain microbials may be perceived as a drawback, especially if a grower is trying to target a variety of pests at one time (Fuxa, 1987; Shapiro-Ilan et al., 2012).

In many systems, another disadvantage to implementing microbial agents in biological pest suppression is that they cost more to use than chemical pesticides. However, unlike arthropod parasitoids, some microbial agents can be produced in vitro, which can substantially decrease the cost. For example, in vitro production systems have been developed for various species of entomopathogenic bacteria, fungi, and nematodes. To date, commercial in vitro production of entomopathogenic viruses has not been accomplished, but research is underway to achieve that goal. In the meantime,

the production of entomopathogenic viruses relies exclusively on in vivo technology. Although commercial entomopathogenic nematodes usually are produced in vitro, some companies still produce nematodes in vivo, which results in higher production costs for labor and insect hosts.

Production efficiency and cost are critical factors affecting the success or failure of commercial ventures involving entomopathogens (Lacey et al., 2001; Shapiro-Ilan et al., 2012). The chapters on entomopathogens review and analyze various factors that affect the production of each group. A number of factors that impact efficiency are shared across the entomopathogen groups, including choice of species or strain, strain stability and improvement, environmental factors (e.g., temperature, humidity, and aeration), inoculation rates, and production densities. Also, regardless of entomopathogen group, media composition is a critical factor for in vitro production, and host species and quality is crucial for in vivo production. Moreover, certain factors pertain only to some groups and may be highly specific. For example, bioreactor design and fermentation parameters only pertain to entomopathogens produced under the liquid in vitro conditions. For the production of heterorhabditid nematodes in liquid culture, recovery at the initiation of molting from the dauer stage is an important issue. The cost of pesticide registration can also be a major consideration prior to commencing production of most entomopathogens, but this issue is generally not relevant for entomopathogenic nematodes because in most countries they are exempt from the registration requirements that apply to other pathogen groups (Ehlers, 2005).

In addition to discussing factors that affect production efficiency and cost, recent advances in production technology are reviewed for each pathogen group. For example, recent advances in the production of entomopathogenic nematodes include the production and infection of infected host cadavers, automated technology for in vivo production, and the use of inbred lines to stabilize beneficial traits in production strains (Shapiro-Ilan et al., 2003, 2010; Bai et al., 2005; Morales-Ramos et al., 2011; see Chapter 10). A recent innovation in fungus production was based on the discovery that *Metarhizium* spp. can produce microsclerotia (compact melanized bodies that conidiate upon rehydration); thus, production technology for these novel propagules has ensued (Jackson and Jaronski, 2012; see Chapter 11). As another example, in Chapter 13, the improvement of baculovirus production through the study of genomics/transcriptomics of insect cell lines is discussed.

The section on entomopathogens offers an analysis of state-of-the-art production in various systems. Considerations for production may vary in different markets, countries, and economies. For example, Chapter 15 presents a perspective on production technology in less industrialized countries. Production technologies used in these situations may not be viable elsewhere because of increased labor costs and a lack of mechanized production systems. However, production ventures in less industrialized countries may face different hurdles, such as reduced levels of capital, infrastructure, or technology (see Chapter 15). Regardless of the production system, one critical factor to the success of all entomopathogen products is formulation. Chapter 14 is devoted entirely to issues related to the formulation of entomopathogens. Formulations are not only required as simple carriers for most microbial control agents, but may also provide other benefits such as improved shelf life, protection from environmental degradation, ease of handling, and enhanced efficacy (see Chapter 14). Clearly, the combination of production and formulation technology is paramount to the successful implementation of entomopathogens in microbial control. Thus, the collection of chapters on microbial control agents brings together the challenges facing the industry and potential solutions on how to enhance commercialization in the future.

1.4 Challenges of mass-producing invertebrates for their products and ecological services

Commercial materials from mass-reared invertebrates consist mostly of silk, honey, wax, dye, and by-products. Silk is mostly produced by culturing the mulberry silk moth, *Bombix mori* L., but other species are also commercially grown to produce silk, including the Chinese Tussah moth, *Antheraea pernyi* (Guénerin-Méneville); the Assam silk moth, *A. assamensis* (Helfer); the tensan silk moth, *A. yamamai* (Guénerin-Méneville); and the eri silk moth *Samia cynthia* (Drury) (Hill, 2009). In addition to the production of silk, the often discarded pupae of the silk moth can be used as a source of food for domesticated animals and people because the pupae have a high nutritional value (Lin et al., 1983; Mishra et al., 2003; Khatun et al., 2005; Longvah et al., 2011). Silk moth production has provided resources for the development of biological control in China. For example, the production of *Trichogramma* spp. parasitoids to control lepidopteran pests are commonly accomplished by using silk moth eggs. Additionally, in vitro production of *Trichogramma* spp. in China is based on the use of pupal silkworm hemolymph (Grenier, 1994). Thus, the biological control industry benefits from another insect production capability. By developing new industries that produce materials from invertebrates, the biocontrol industry also can be advanced.

Ecological services provided by mass-reared invertebrates include human food and animal feed, pollination, waste decomposition, soil restoration, and biological control. Commercially produced insects sold for feed include the house cricket, the rusty red roach, *Blatta lateralis* Walker; the greater wax moth, *Galleria mellonella* L.; the butter worm, *Chilecomadia moorei* Silva; the mealworm, the super worm, *Zophobas morio* F.; the black soldier fly, and the house fly (Finke, 2002, 2013). Several of these species have been studied for use as poultry feed (Calvert et al., 1969; Klasing et al., 2000; Ramos-Elorduy et al., 2002; Zuidhof et al., 2003; Anand et al., 2008; Ijaiya and Eko, 2009; De Marco et al., 2015; Dabbou et al., 2018; Józefiak et al., 2018; Moula et al., 2018; Benzertiha et al., 2019). Cultured insects have also been investigated for use as feed in aquaculture (Bondari and Sheppard, 1987; Ng et al., 2001; Fasakin et al., 2003; Barroso et al., 2014; Gasco et al., 2018; Lock et al., 2018; Ferrer Llegostera et al., 2019). Moreover, some of the commercially produced insect species have even been proposed as potential food for humans (Gordon, 1998; Ramos-Elorduy, 1998; DeFoliart, 1999; Gahukar, 2011, 2016; van Huis et al., 2014; Tang et al., 2019). Insects contain adequate levels of most nutrients for vertebrate nutrition including proteins, lipids, minerals, and B-complex vitamins (Goulet et al., 1978; Ramos-Elorduy, 1997; Bukkens, 1997; DeFoliart, 1992; Barker et al., 1998; Finke, 2002, 2013, 2014, 2015; Oonincx and Dierenfeld, 2012). Insect production is considered to have less impact on the environment and be more sustainable than other sources of animal food such as cattle, pork, and poultry (Ramos-Elorduy, 1997; Carlsson-Kanyama, 1998; Oonincx et al., 2010; Premalatha et al., 2011; Oonincx and de Boer, 2012; Miglietta et al., 2015; Gahukar, 2016; Halloran et al., 2016; Smetana et al., 2016). Insects convert food more efficiently (Capinera, 2004; Oonincx et al., 2010; Gahukar, 2011), produce fewer greenhouse gas emissions (Oonincx et al., 2010; Oonincx and de Boer, 2012), and require less water (Miglietta et al., 2015) and space (Oonincx et al., 2010) than cattle, pigs and poultry, and insect production requires less energy per kg of biomass produced (Oonincx et al., 2010; Premalatha et al., 2011). Although, most current methods of insect mass production for feed and food are still in their infancy, production capabilities are increasing rapidly to provide a viable alternative to conventional animal protein sources. The potential of insects as a sustainable source of food for the future is explored further in Chapter 18.

A few invertebrate species are produced commercially for pollination and soil restoration. Traditionally, pollination of high-value crops has been accomplished by managing the honey bee, *Apis melifera* L. (O'Toole, 2008). The culture and use of solitary bees have increased recently in North America, however. One example is the leaf cutting bee, *Megachile rotunda* F., which is used in alfalfa pollination (Stephen, 2003). Other species cultured for alfalfa pollination include the alkali bee, *Nomia melanderi* Cockerell (Cane, 2008). The blue orchard bee, *Osmia lingaria* Say, is used as a pollinator in many high-value crops, for example, almonds, apples, pears, and cherries (Bosch and Kemp, 2002; Torchio, 2003; Cane, 2005; Bosch et al., 2006). The culture and use of solitary bees for pollination are reviewed in Chapter 19. For soil restoration, various earthworms are produced commercially, including *Aprrectodea longa* (Ude), *A. caliginosa* (Savigny), *Allobophora chlorotica* (Savigny), and *L. terrestris* (Lowe and Butt, 2005). Earthworm species also have been commercialized for fish bait and fish feed such as *Lumbricus terrestris* L., *L. rubellus* Hoffmeister, *Eudrilus eugeniae* (Kinberg), and *Eisenia foetida* (Savigny) (Harper and Greaser, 1994; Mason et al., 2006). Culture techniques and applications for earthworms are discussed in Chapter 20.

In conclusion, the challenges of mass-producing beneficial organisms, particularly arthropods and pathogens for biological control, are addressed in this book. Production technologies for beneficial organisms often are based on systems originally developed to mass-produce insects for pest management, but they have improved with advances in related science and technology. The systems created by these innovations have many comparable production processes, although each is unique to the biology of the species being mass-produced. The new methods and materials incorporated into a production system for one species often can be adapted for use with another, thereby advancing the entire field of mass producing beneficial organisms. Thus, the primary purpose of this book is to assemble examples of production systems for arthropods, pathogens, and other beneficial organisms that can be compared and adapted to develop efficient mass production systems.

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Chapter 2

Production of coleopteran predators

Eric W. Riddick

Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States

2.1 Introduction

2.1.1 Aims of this chapter

This chapter reviews the scientific literature to spotlight research on the production of coleopteran predators, with a primary focus on lady beetles (family Coccinellidae). Mass production is necessary for the permanent establishment of predators, periodic colonization of predators in an area to augment existing populations, and inundative releases for short-term control of a pest (Etzel and Legner, 1999). Mass production will also benefit researchers interested in testing the side effects of toxins on beneficial, nontarget arthropods (Martos et al., 1992; Li et al., 2011). Finally, mass production could also benefit scientists interested in restoring populations of rare, threatened, or endangered coleopteran predators (Harmon et al., 2007; Gwiazdowski et al., 2011). The main emphasis of this chapter is on reviewing the published literature over the last few decades to develop new and creative insights on predator production for applied biological control. Transfer of these insights to the biological control industry, especially the natural enemy industry, should be a major outcome of this work. Why dedicate an entire chapter solely to predatory beetles? One of the most successful biological control programs involved the use of a lady beetle, namely the vedalia beetle *Rodolia cardinalis* (Mulsant) to control cottony cushion scale *Icerya purchasi* Maskell in California citrus groves in 1889 (Caltagirone and Doutt, 1989). The outstanding performance of *R. cardinalis* led to a worldwide fascination with lady beetles and other natural enemies that could provide control of crop pests on a spectacular scale (Sawyer, 1996). Today, one challenge to the greater use of lady beetles and other less-heralded coleopteran predators in applied biological control is to create cost-effective techniques to rear and stockpile (store) species. Many of the current rearing methods continue to depend on a tritrophic system of rearing; the host plant, natural prey (herbivorous pest), and predator. This system is labor intensive and not cost-effective. Therefore, the aim of this work is to highlight what is known about rearing predatory coleopterans and provide ideas and suggestions that might stimulate more advances in this important field of study.

2.1.2 Predatory beetles in culture

Predatory beetles (order Coleoptera) have a long history of importance as natural enemies of plant-feeding insects and mites in natural and managed ecosystems. Many coleopteran predators, especially those in the family Coccinellidae (representing the lady beetles, ladybird beetles, or ladybugs) are used with moderate success in managing pest populations throughout the world (Hodek and Honěk, 1996; Obrycki and Kring, 1998; Hagen et al., 1999; Hodek and Evans, 2012). Some of the coleopteran predators covered in this chapter are or have been commercially available for biological control of crop pests. van Lenteren (2012) provides a list of species available at present or in the past with information on the region of the world the insects have been (for augmentative biological control), estimated year of first use, and the market value (small, medium, large) of each species. Many of these taxa have been subjects of research on nutritional ecology and rearing as it relates to biological control (Hagen, 1987; Hodek, 1996; Thompson and Hagen, 1999; van Lenteren, 2003). In this chapter, a list of exemplary coleopteran predators that have been subjects of rearing related research is provided (Table 2.1).

2.1.3 Overview of the content

The coverage of this chapter is in favor of coccinellids, because most rearing-related work has historically focused on this group of predatory beetles. The most detailed section of this book chapter addresses the food of predatory beetles.

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TABLE 2.1 Predatory Coleoptera as subjects of mass production research.

Family	Species[a]	Natural/target prey	References
Carabidae	*Abax parallelepipedus* Piller & Mitterpacher	Snails, slugs	Symondson (1994)
Carabidae	*Calosoma sycophanta* (L.)	Gypsy moth larvae, pupae	Weseloh (1998)
Carabidae	*Lebia grandis* Hentz[b]	Colorado potato beetle immatures	Weber et al. (2006), Weber and Riddick (2011)
Carabidae	*Poecilus chalcites* (Say)	Dipterans, lepidopterans	Lundgren et al. (2005)
Cleridae	*Thanasimus dubius* (F.)	Bark beetles	Reeve et al. (2003), Costa and Reeve (2012)
Cleridae	*Thanasimus formicarius* (L.)	Bark beetles	Faulds (1988), Lawson and Morgan (1992)
Coccinellidae	*Adalia bipunctata* (L.)[a]	Aphids	Kariluoto et al. (1976), Bonte et al. (2010), Lommen et al. (2019)
Coccinellidae	*Brumoides suturalis* (F.)	Scales, mealybugs	Bista et al. (2012)
Coccinellidae	*Chilocorus bipustulatus* (L.)[a]	Scales	Henderson et al. (1992)
Coccinellidae	*Chilocorus nigritus* (F.)[a]	Scales	Hattingh and Samways (1993)
Coccinellidae	*Cleobora mellyi* (Mulsant)	Psyllids, chrysomelids	Bain et al. (1984)
Coccinellidae	*Clitostethus arcuatus* (Rossi)[a]	Whiteflies	Mota et al. (2008); Yazdani and Zarabi (2011)
Coccinellidae	*Coccinella septempunctata* L.[a]	Aphids	Sarwar and Saqib (2010), Cheng et al. (2018)
Coccinellidae	*Coccinella undecimpunctata* L.	Aphids	Farag et al. (2011)
Coccinellidae	*Coleomegilla maculata* (DeGeer)	Aphids, lepidopterans, coleopterans	Atallah and Newsom (1966), Silva et al. (2010), Riddick and Wu (2015a,b), Rojas et al. (2016)
Coccinellidae	*Cryptolaemus montrouzieri* Mulsant[a]	Scales, mealybugs	Heidari and Copland (1993), Attia et al. (2011), Maes et al. (2014), Li et al. (2021)
Coccinellidae	*Delphastus catalinae* (Horn)[a]	Whiteflies	Pickett et al. (1999), Kutuk and Yigit (2007)
Coccinellidae	*Diomus terminatus* (Say)	Aphids	Tifft et al. (2006)
Coccinellidae	*Eriopis connexa* (Germar)	Aphids, lepidopterans	Martos et al. (1992), Silva et al. (2009, 2013b)
Coccinellidae	*Harmonia axyridis* (Pallas)[a]	Aphids	Dong et al. (2001), Sighinolfi et al. (2008), Ricupero et al. (2020)
Coccinellidae	*Hippodamia convergens* Guerin[a]	Aphids	Hussein and Hagen (1991), Kato et al. (1999)
Coccinellidae	*Hippodamia variegata* (Goeze)[a]	Aphids	van Lenteren (2012), Sayed and Arnaouty (2016)
Coccinellidae	*Menochilus sexmaculatus* (F.)	Aphids, scales, psyllids	Khan and Khan (2002)
Coccinellidae	*Nephus includens* (Kirsch)[a]	Mealybugs	Canhilal et al. (2001)
Coccinellidae	*Olla v-nigrum* Mulsant	Aphids	Silva et al. (2013a)

(*Continued*)

TABLE 2.1 (Continued)

Family	Species[a]	Natural/target prey	References
Coccinellidae	*Propylea dissecta* (Mulsant)	Aphids	Omkar and Pathak (2009)
Coccinellidae	*Propylea japonica* (Thunberg)	Aphids	Hamasaki and Matsui (2006), Wang et al. (2008), Liu et al. (2020)
Coccinellidae	*Rhyzobius lophanthae* (Blaisdell)[a]	Scales	Stathas (2000)
Coccinellidae	*Rodolia cardinalis* (Mulsant)[a]	Scales	Matsuka et al. (1982), Grafton-Cardwell et al. (2005)
Coccinellidae	*Sasajiscymnus tsugae* (Sasaji & McClure)	Adelgids	Flowers et al. (2007), Conway et al. (2010)
Coccinellidae	*Stethorus gilvifrons* (Mulsant)	Mites	Ebrahimifar et al. (2020b), Ebrahimifar et al. (2020a)
Coccinellidae	*Stethorus punctillum* Weise[a]	Mites	Riddick et al. (2011a,b)
Colydiidae	*Dastarcus helophoroides* (Fairmaire)[b]	Cerambycid larvae, pupae	Ogura et al. (1999), Gao et al. (2019)
Histeridae	*Carcinops pumilio* (Erichson)[a]	Dipterans	Achiano and Giliomee (2006, 2007)
Rhizophagidae	*Rhizophagus grandis* Gyllenhal	Bark beetles	Couillien and Gregoire (1994)
Staphylinidae	*Holobus flavicornis* (Lacordaire)[a]	Mites	van Lenteren (2012)
Staphylinidae	*Aleochara bilineata* (Gyllenhal)[a]	Dipterans	Whistlecraft et al. (1985)
Staphylinidae	*Creophilus maxillosus* L.	Dipterans	Greene (1996)
Staphylinidae	*Dalotia coriaria* (Kraatz)[a]	Dipterans, thrips, coleopterans	Birken and Cloyd (2007)
Trogossitidae	*Temnochila virescens* (F.)	Bark beetles	Lawson and Morgan (1992)
Trogossitidae	*Trogossita japonica* Reitter	Cerambycid larvae	Ogura and Hosoda (1995)

[a]*Species sold commercially in the past or present for biological control of pests (see van Lenteren, 2012).*
[b]*Larvae are ectoparasitoids of host pupae.*
This table was updated from Table 2.1 in Ch. 2, 1st ed.

Knowledge of the nutritional requirements of a predator is central to devising methods to produce the insect in sufficient quantities for biological control. Feeding preferences of predators, when presented with natural prey, are also mentioned. Evidence for feeding on factitious food/prey (i.e., alternatives to natural prey) and artificial diets are provided.

The next section of this chapter will review the importance of rearing density (population density) on the production of predatory beetles. Two critical topics of concern are the effects of crowding and cannibalism on production. Next, the design of oviposition substrates and enclosures or rearing cages that support the most efficient population growth will be examined. Issues of rearing scale and the possibility of large-scale production of predators will also be reviewed. Controlling and maintaining temperatures inside enclosures that maximize the growth of predatory beetles are very important. Methods that scientists use to estimate the optimum temperature for population growth will be discussed. The next section of this chapter concerns checks and balances to ensure that unwanted pathogens and parasites do not infiltrate cultures. Other concerns such as preventing the deterioration of cultures through inbreeding and other genetic means will be addressed. The importance of assessing the health (quality) of cultures at the preshipment, in-shipment, and prerelease stages will be considered. The final section will conclude this chapter, synthesize data, and provide recommendations for future research.

2.2 Foods and production of predators

2.2.1 Feeding preferences and natural prey

Feeding preferences of coleopteran predators vary greatly. Feeding preferences in the context of this review refer to the innate restrictions, or lack of, that predators display toward potential prey. Scientists often group species into categories of polyphagous, oligophagous, and monophagous, indicating broad to narrow feeding preferences. Truly monophagous species, which specialize in one prey species or genus, are rare in nature. Most coleopteran predators likely fall along a continuum between oligophagous and polyphagous. Some reportedly oligophagous predators include a carabid *Lebia grandis* Hentz (Weber et al., 2006; Weber and Riddick, 2011), coccinellids *Sasajiscymnus tsugae* (Sasaji & McClure) (Flowers et al., 2007; Jetton et al., 2011), *Stethorus punctillum* Weise (Riddick et al., 2011a,b), *Stethorus gilvifrons* (Mulsant) (Ebrahimifar et al., 2020b), *R. cardinalis* (Matsuka et al., 1982; Grafton-Cardwell et al., 2005) and *Clitostethus arcuatus* (Rossi) (Mota et al., 2008; Yazdani and Zarabi, 2011). Most coleopteran predators used previously in biological control, and reared in the laboratory, are moderately polyphagous (Table 2.1).

Researchers often use the terms essential and alternative (nonessential) prey when they refer to the food preferences of coccinellids; essential prey are required for multigenerational development and reproduction whereas alternative prey typically sustain the lifespan of a predator during shortages of essential prey (Hodek and Honěk, 1996). Intuitively, the structure of the mouthparts, digestive tract, and the spectrum of enzymes in the digestive tract of a predator should correlate with feeding preferences, in a general sense. Some correlations do exist but at the family or genus level rather than the species level in coccinellids (Minelli and Pasqual, 1977; Samways et al., 1997). Giorgi et al. (2009) provide a review of the purported evolution of food preferences in coccinellids.

Researchers have experimented with rearing coccinellids and other predatory beetles for biological control over the years. Several coccinellids, staphylinids and a histerid have been commercially available (Table 2.1). The technology of producing these predators remains in an infant stage; rearing on natural prey is commonplace. Nevertheless, researchers have tried to rear several coccinellid and noncoccinellid coleopteran predators on artificial diets with or without arthropod components.

Most rearing-related research on coleopteran predators to date has involved coccinellids. Detailed reviews of the feeding preferences of coccinellids are available for species that prefer aphids (Obrycki et al., 2009), scales, mealybugs, whiteflies, and psyllids (Hodek and Honěk, 2009), mites (Biddinger et al., 2009), and nonhemipteran insects, such as other coleopterans and lepidopterans (Evans, 2009). By far, most of this work has concentrated on coccinellids that prefer aphids (aphidophagous) as prey, despite the belief that species that utilize other insects as prey (such as scales) are more effective biocontrol agents (Magro et al., 2002). Some aphidophagous coccinellids are polyphagous and can reproduce on other insects, besides aphids. Coccinellids that prefer scales, mealybugs, whiteflies, psyllids, or mites (rather than aphids) apparently are more restricted in their prey preferences (Hodek and Evans, 2012). Researchers are currently testing various techniques to increase mass production of the coccinellid *S. tsugae* on woolly adelgids in several laboratories in eastern North America for augmentative releases into forests to suppress populations of the hemlock woolly adelgid (Cohen et al., 2008; Conway et al., 2010). Development of methods of rearing *S. tsugae* using natural prey or target prey is not cost-effective over the long term due to the difficulties of developing effective rearing methods for the prey species. Alternative (i.e., factitious) prey could provide a way to expedite and streamline the rearing of these important coccinellids. A factitious prey species could be chosen because is easier or less expensive to rear than natural or target prey of coleopteran predators.

2.2.2 Feeding on factitious foods and plant products

Factitious prey/foods are typically insects, mites, or crustaceans not consumed under natural conditions that support the development and reproduction of predators (although often at suboptimal levels) in lieu of natural or target prey. Researchers have presented factitious prey/food to predators in various forms; live, irradiated, frozen or lyophilized. Lepidopteran eggs represent a nutrient-rich food for some generalist coccinellids (Herrera, 1960; Riddick, 2009; Sun et al., 2017), but not others (Michaud and Qureshi, 2005; Hodek and Evans, 2012). Abdel-Salam and Abdel-Baky (2001) fed the multicolored Asian lady beetle *Harmonia axyridis* (Pallas) larvae and adults fresh versus frozen eggs of the grain moth, *Sitotroga cerealella* Olivier; fresh *S. cerealella* eggs were more nutritious or beneficial for predator development and reproduction. Specty et al. (2003) found that *Ephestia kuehniella* (Zeller) eggs contained a greater percentage of amino acids and lipids compared to the pea aphid *Acyrthosiphum pisum* (Harris), a natural prey of *H. axyridis*; pea aphids contained a greater percentage of glycogen. The food intake of *H. axyridis* was threefold greater when feeding on pea aphids than *Ephestia* eggs. According to Specty et al. (2003), *Ephestia* eggs had a positive effect on *H. axyridis* growth and reproduction (see Table 2.2).

TABLE 2.2 Coccinellid predators[a] and effects of factitious food on life history parameters.

Lady beetle	Factitious food	Results	References
Cryptolaemus montrouzieri (larvae, adults)	*Ephestia kuehniella* eggs	Development (↓), fecundity (↓), longevity (✓)	Attia et al. (2011)
Harmonia axyridis (larvae, adults)	*E. kuehniella* eggs	Survival (↑), size (↑), fecundity (↑)	Specty et al. (2003)
Hippodamia convergens (larvae, adults)	*E. kuehniella* eggs	Development (↑), oviposition period (✓), fecundity (✓), longevity (✓)	Kato et al. (1999)
Propylea japonica (larvae, adults)	*E. kuehniella* eggs	Survival (✓), fecundity (↓)	Hamasaki and Matsui (2006)
Adalia bipunctata (larvae, adults)	*E. kuehniella* eggs plus pollen	Development (✓), survival (✓)	De Clercq et al. (2005a)
	E. kuehniella eggs plus pollen	Development (↓), oviposition (↓), fecundity (↓)	Jalali et al. (2009b)
A. bipunctata (larvae)	*E. kuehniella* eggs plus pollen	Development (↑), survival (✓), size (✓), consumption (↑)	Jalali et al. (2009a)
A. bipunctata (larvae, adults)	*E. kuehniella* eggs plus pollen	Development (↓), survival (↓), size (↓), fecundity (↑), egg hatch (↓), longevity (✓)	Bonte et al. (2010)
	Brine shrimp cysts (eggs) plus pollen	Development (↓), survival (↓), size (↓), fecundity (✓), egg hatch (↓), longevity (↑)	Bonte et al. (2010)

[a]*Data on noncoccinellid beetles included in text only.*

Ettifouri and Ferran (1993) and Ferran et al. (1997) found that rearing *H. axyridis* on *Ephestia* eggs over many generations could reduce the capacity of larvae to intensely search for and recognize chemical cues (tracks) of its prey, the pea aphid, *A. pisum*. Caution must be used when rearing predators solely on factitious prey without a period of exposure to natural prey prior to releasing them for biological control.

Attia et al. (2011) proved that it was possible to rear larvae and adults of the mealybug destroyer *Cryptolaemus montrouzieri* Mulsant on *E. kuehniella* eggs. *C. montrouzieri* larvae developed faster on *E. kuehniella* eggs than on mealybug *Planococcus citri* (Risso) eggs (Table 2.2). Although fecundity was 33% less (on average), adult longevity was approximately the same for females fed *Ephestia* eggs in place of *P. citri* eggs. Pilipjuk et al. (1982) succeeded in rearing *C. montrouzieri* on *S. cerealella* eggs. However, mealybug ovisacs or an extract of mealybugs or their ovisacs were required to stimulate oviposition. Heidari and Copland (1993) found that honeydew was a food source or at least an arrestant for *C. montrouzieri* larvae and adults. Adults spent more time foraging on portions of leaves coated with honeydew but spent less time foraging on clean surfaces. However, egg production was very low on a diet of honeydew alone. Research on identifying more effective factitious foods and rearing substrates for *C. montrouzieri* is ongoing (Maes et al., 2014; Li et al., 2021).

When offered *Ephestia* eggs, the convergent lady beetle *Hippodamia convergens* Guerin-Meneville took a few days longer to complete development than when fed aphids, *Schizaphis graminum* (Rondani) and *Brachycaudus schwartzi* Börner (Kato et al., 1999). Other biological parameters relating to adult lifespan and reproduction were comparable for predators fed aphids (*S. graminum*) versus *Ephestia* eggs (Table 2.2). Hamasaki and Matsui (2006) found that larvae and adults of a coccinellid *Propylea japonica* (Thunberg) developed and reproduced on *Ephestia* eggs in place of pea aphids; however, fecundity was lower for adults reared on the moth eggs. Lima et al. (2018) discovered that *Ephestia* eggs could be used for rearing larvae but not adults of a coccinellid *Brumoides foudrasii* (Mulsant), a predator of mealybugs in South America.

The potential of dipteran eggs and larvae as factitious food/prey for coccinellids has been explored in a few studies. Riddick et al. (2014b) used muscid (*Musca domestica* L.) eggs to rear *Coleomegilla maculata* (*DeGeer*) larvae to adults; adult females produced viable offspring. Schultz et al. (2017) utilized drosophilid (*Drosophila melanogaster* Meigen) larvae as factitious prey to successfully rear *C. maculata* larvae to adults in the laboratory. *C. maculata* adults produced healthy offspring.

Researchers are revealing the benefits of combining insect and plant products for improving the fitness of natural enemies (Smith, 1960, 1965; Lundgren, 2009, 2010; Farag et al., 2011; Riddick et al., 2014b; Riddick and Wu, 2015a, b; Sayed and Arnaouty, 2016; D'Ávila et al., 2017; Ebrahimifar et al., 2020b). Some predators can compensate for a less than an optimal diet of factitious prey by feeding on plant protein, carbohydrates, and sterols (pollen) when it is available. Consumption of plant material during periods of prey shortage is a survival strategy of some predators (Berkvens et al., 2008, 2010). De Clercq et al. (2005b) discovered that supplementing *Ephestia* eggs with frozen moist bee pollen, rather than natural prey (live pea aphids), allowed for adequate egg hatch, development, and survival of *Adalia bipunctata* L. larvae. *Ephestia* eggs plus fresh bee pollen combination is as nutritionally suitable as aphids for *A. bipunctata* larval development but not for reproduction (Jalali et al., 2009a,b). Bonte et al. (2010) found that *A. bipunctata* development, as well as reproduction (fecundity), improved when fed a mixture of moist bee pollen plus *E. kuehniella* eggs rather than pea aphids (see Table 2.2).

Brine shrimp (*Artemia* spp.; Branchiopoda), a product of the aquaculture industry, is relatively inexpensive to produce in comparison to *Ephestia* moth eggs (De Clercq et al., 2005a). Hongo and Obayashi (1997) were among the first to test *Artemia* eggs as factitious food for *H. axyridis*. They discovered that *Artemia salina* (L.) eggs alone supported the development of larvae, but not adult emergence, which was just 18%. When combining *A. salina* eggs with sucrose and Y2A (Brewer's yeast, Asahi Breweries, Japan), adult emergence improved significantly (61%). The weight of adult females was about the same regardless of whether they fed on *A. salina* eggs alone or eggs with the additives (Hongo and Obayashi, 1997). A combination of brine shrimp powder and synthetic bee pollen supported the development and reproduction of *C. maculata* in laboratory experiments and toxicity bioassays (Lundgren and Weber, 2010; Pilorget et al., 2010; Li et al., 2011; Lundgren et al., 2011; Weber and Lundgren, 2011). However, these studies did not compare the benefits of using brine shrimp versus natural prey or *Ephestia* eggs as food for *C. maculata*. Bonte et al. (2010) compared the benefits of brine shrimp eggs (cysts) plus moist bee pollen versus natural prey (pea aphids) on the life history of *A. bipunctata* (see Table 2.2). They found that development time increased, size decreased, but longevity increased when predators consumed brine shrimp eggs rather than pea aphids. More recent research has supported the use of brine shrimp eggs alone, or in combination with *Ephestia* eggs, as factitious food for lady beetles (Riddick et al., 2014a; Riddick and Wu, 2015a,b; Rojas et al., 2016; Seko et al., 2019; Ricupero et al., 2020).

Birken and Cloyd (2007) observed the feeding preferences of the staphylinid *Dalotia coriaria* (Kraatz), a predator of fungus gnats and thrips in greenhouses. They discovered that larvae and adults consume oatmeal in the presence or absence of fungus gnat larvae in laboratory arenas. Oatmeal can serve as an inexpensive supplemental food in the rearing of *D. coriaria*, which is commercially available for the control of fungus gnats in greenhouses.

2.2.3 Feeding on artificial diets

The utilization of an artificial diet may represent a step toward more cost-effective rearing of coccinellids and other coleopteran predators. Artificial diets have been classified by the identification of chemicals within them. These include holidic diet, in which all constituents are known in the chemical (molecular) structure; meridic diet, in which most of the constituents are known chemically, and oligidic diet, in which a few of the constituents are known chemically (Vanderzant, 1974). An alternative system of classification distinguishes artificial diets based on the presence or absence of insect components (i.e., tissues, hemolymph, cells, protein, amino acids, etc.) within them (Grenier and De Clercq, 2003; Grenier, 2009). The latter system is preferred in this chapter. However, "insect components" is expanded to "arthropod components" to reflect the usage of protein from crustaceans in artificial diets.

Artificial diets containing arthropod components—Artificial diets that contain insect matter (tissues, protein, cells, etc.) are useful when predators need chemical cues and other feeding stimulants found in live prey (De Clercq, 2004). There has been only limited success in rearing predatory beetles with artificial diets containing arthropod components. To date, most research has centered on aphidophagous coccinellids. Smirnoff (1958) was one of the first to formulate artificial diets (with arthropod components) for coccinellids. He reported that an artificial medium that could be used to rear adults was essentially composed of cane sugar, agar, royal jelly, honey, and pulverized, dry insects (natural prey of the predator species in rearing). To rear larval stages, he supplemented this medium with royal jelly and beef jelly. Although 19 species (in the genera *Coccinella*, *Harmonia*, *Thea*, *Rhizobius*, *Rodolia*, *Exochomus*, *Scymnus*, *Stethorus*, *Chilocorus*, *Clitostethus*, *Pharoscymnus*, and *Mycetaea*) were reared on these two media, Smirnoff (1958) did not present any data on the effects of these media on predator development and reproduction. He does mention that gravid females would not produce eggs when fed the media inside the confines of a Petri dish; placement of females in larger cages containing twigs of a plant that the predator forages on under natural conditions induced oviposition. This study shows that several coccinellids (*Mycetaea tafilaletica* Smirnoff, *Scymnus kiesenwetteri* Mulsant, *Scymnus pallidivestis*

Mulsant, and *Thea vigintiduopunctata* L.) lived from 3 to 5 times longer when fed the media rather than natural prey (Smirnoff, 1958). This study also suggests that plant volatiles can provide oviposition cues for coccinellids.

Sarwar and Saqib (2010) attempted to rear the aphidophagous 7-spot ladybird *Coccinella septempunctata* L. using an artificial diet like the one used by Smirnoff (1958). Their diet consisted of cane sugar, honey, protein hydrolysate, royal jelly, yeast, and pulverized, dried, mustard aphid (*Lipaphis erysimi* Kaltenbach). In comparison to natural prey (mustard aphid), the artificial diet was substandard; a low percentage of larvae completed development and females produced much fewer eggs when fed the artificial diet (Table 2.3).

The aphidophagous coccinellid *P. japonica* completed development when fed crude artificial diets consisting of powdered formulations of defatted yellow mealworm (*Tenebrio molitor* L.) pupae with yeast and sugar or housefly (*M. domestica*) larvae with yeast and sugar versus soybean aphid *Aphis glycines* Matsumura (Wang et al., 2008). However, when compared to a diet of soybean aphids, the two powdered diets had negative effects on larval development and body size (Table 2.3). Liu et al. (2020) developed an improved artificial diet, based on powdered noctuid moth *Mythimna separata* (Walker) larvae, for *P. japonica* larval development to the adult stage. The authors did not indicate whether this artificial diet supported *P. japonica* reproduction.

TABLE 2.3 Coccinellid predators[a] and effects of artificial diets on life history parameters.

Lady beetle	Artificial diet—protein base	Results	References
Adalia bipunctata (larvae, adults)	Ground beef, beef liver, chicken egg yolk plus pollen	Development (↑), size (↓), survival (↓), fecundity (↓), longevity (✓)	Bonte et al. (2010)
	Ground beef, beef liver, chicken egg yolk plus pollen, brine shrimp (*Artemia*) eggs	Development (↑), size (↓), survival (✓), fecundity (↓), longevity (✓)	Bonte et al. (2010)
Chilocorus nigritus (larvae, adults)	Honeybee brood (powdered)	Development (↑), adult emergence (↓), size (↓), fecundity (↓)	Hattingh and Samways (1993)
Clitostethus arcuatus (larvae, adults)	Yeast, honey, pollen	Fecundity (↓), fertility (↓), sex ratio (✓), longevity (↑)	Yazdani and Zarabi (2011)
Coccinella septempunctata (larvae, adults)	Yeast, protein hydrolysate, aphids (powdered)	Development (↑), survival (↓), oviposition (↓)	Sarwar and Saqib (2010)
Coleomegilla maculata (larvae)	Yeast plus *Ephestia* eggs	Development (✓), survival (✓), size (✓)	Silva et al. (2010)
Eriopis connexa (larvae)	Cat food plus *Ephestia* eggs	Development (↑), adult emergence (↓), size (↓),	Silva et al. (2009)
Harmonia axyridis (larvae, adults)	Chicken egg yolk, chicken liver, casein hydrolysate	Development (↑), survival (✓), size (↓), oviposition (↓)	Dong et al. (2001)
	Chicken whole egg, chicken liver, casein hydrolysate	Development (↑), survival (✓), size (↓), oviposition (↓)	Dong et al. (2001)
	Pork liver, amino acid solution	Development (↑), adult emergence (↓), size (↓), preoviposition period (↑), fecundity (↓)	Sighinolfi et al. (2008)
Menochilus sexmaculatus (larvae, adults)	Chicken liver, powdered	Development (↑), survival (↓), fecundity (↓), longevity (↓)	Khan and Khan (2002)
Propylea japonica (larvae, adults)	Housefly (powdered)	Development (↑), adult emergence (✓), size (↓)	Wang et al. (2008)
	Mealworm (powdered)	Development (↑), adult emergence (✓), size (↓)	Wang et al. (2008)

Symbols indicate that diets have no significant effect (✓), increase (↑), or decrease (↓) a life parameter when compared against a control (usually natural prey).
[a]*Data on noncoccinellid beetles included in text only.*

Rojas et al. (2016) tested the usefulness of an artificial diet supplemented with 7% powdered *T. molitor* pupae to rear *C. maculata*. The powder-based diet enhanced *C. maculata* larval development and survival to the adult stage and improved adult fecundity and offspring viability when compared to two insect-free, semisolid meridic diet.

Silva et al. (2009) found that an artificial diet containing a commercial cat food did not support the development of the aphidophagous coccinellid *Eriopis connexa* (Germar) in the adult stage. Note that simply adding eggs of the moth *E. kuehniella*, frozen for one day, to this artificial diet resulted in a development rate of 72% from the larval to adult stage. In a similar study, Silva et al. (2010) reared *C. maculata* on a yeast-based artificial diet plus moth eggs, *E. kuehniella* in comparison to *Ephestia* eggs alone, *Spodoptera frugiperda* (J. E. Smith) eggs alone, or aphids *S. graminum* alone (Table 2.3). Predators took less time to complete larval development when fed aphids rather than the other food treatments, but total development from larva to adult stage did not differ between food treatments. The percentage of larvae surviving to the adult stage was high, beyond 85% (mean value) in all food treatments, with no significant differences between any of the treatments. *C. maculata* males and females fed the artificial diet with *Ephestia* eggs weighed more than individuals fed *Ephestia* eggs alone. Adult males and females fed the artificial diet with *Ephestia* eggs were not heavier than individuals fed aphids alone (Silva et al., 2010). Wipperfürth et al. (1987) examined the effect of color morphs of the green peach aphid, *Myzus persicae* (Sulzer) on egg production by the convergent lady beetle *H. convergens*. Female *H. convergens* were fed an artificial diet, *ad lib*, and less than optimal quantities of wingless virginoparous versus winged gynoparous forms of *M. persicae*. Females failed to produce eggs when fed the artificial diet, which consisted of an 85:5:10 mixture of protein (lyophilized potato tuberworm, *Phthorimaea operculella* (Zeller): yeast autolysate: honey). Females produced more eggs when fed wingless virginoparous *M. persicae*.

Matsuka and Okada (1975) evaluated a series of artificial food sources to support the development of *H. axyridis* larvae. These included yeast, chicken liver, banana, royal jelly, pollen, and dried milk. The addition of pulverized drone honeybee (*Apis mellifera* L.) powder to these diets slightly enhanced diet efficacy. In a follow-up study, a diet consisting of drone honeybee brood was suitable for *H. axyridis* larval development. After fractionating the diet, a cationic fraction, soluble in water, was determined to be essential for larval development. In a subsequent study, potassium was found to be important for successful larval development (Matsuka and Takahashi, 1977). Niijima et al. (1997) experimented with rearing *H. axyridis* on a diet consisting of powdered honeybee brood. Approximately 90% and 80% of *H. axyridis* larvae fed powdered honeybee versus powdered aphid *Lachnus tropicalis* (van der Goot), respectively, reached the adult stage. Development time averaged 19 and 20 days for individuals fed powdered honeybee versus powdered aphids. However, adults had lower body weight when reared on honeybee. Unfortunately, Niijima et al. (1997) did not report the effects of the honeybee diet on fecundity or longevity of *H. axyridis* adults.

In laboratory experiments, Bonte et al. (2010) fed the two-spot ladybird, *A. bipunctata*, another aphidophagous species, an artificial diet based on beef and chicken protein with pollen and brine shrimp cysts (Table 2.3). Development time increased, body size decreased, and fecundity decreased in comparison to natural prey (pea aphids). Larval survival rate and adult longevity were unaffected by the artificial diet in relation to natural prey.

A partially coccidophagous coccinellid, *Cleobora mellyi* Mulsant, was introduced into New Zealand from Australia for biological control of the eucalyptus tortoise beetle *Paropsis charybdis* Stål, a major defoliator of *Eucalyptus* spp. in forests (Bain et al., 1984). An experiment compared freeze-dried pork liver, potato tuberworm larvae, or cerambycid larvae (Huhu grubs) as food for rearing *C. mellyi* larvae. Predators completed development on all three foods, but development was fastest on the tuberworm diet. Note that adults produced from all three foods still required feeding on natural prey (i.e., scales, psyllids, aphids) for approximately a week before any oviposition occurred.

Chilocorus beetles are important predators of scale insects. Two artificial diets, one for adults and the other for larvae, were the best of several ones for maintaining *Chilocorus nigritus* (F.) in the laboratory during shortages of natural prey (Hattingh and Samways, 1993). The best diet for maintenance of *C. nigritus* adults contained powdered honeybee brood (83.5%), royal jelly (1.7%) and glucose (4.5%). The diet considered best for *C. nigritus* larvae contained powdered honeybee brood (55%), royal jelly (3%), honey (4.5%), pulverized Oleander scale *Aspidiotus nerii* Bouché (3%), sucrose (4%), glucose (9%), and fructose (11%). Both diets contained the same percentages of Wheat germ, Brewer's yeast, and ascorbic acid. However, both were not as suitable as natural (or target) prey for culturing this predator over consecutive generations. Predators fed artificial diets rather than scales took longer to develop, were smaller in size, and less fecund (Table 2.3).

Henderson et al. (1992) tested the potential of using freeze-dried, then powdered artificial diets to rear three *Chilocorus* species. The main proteinic constituents in these diets were pupae of the light brown apple moth *Epiphyas postvittana* Walker, pupae of the German yellowjacket wasp *Vespula germanica* (F.) or larvae/pupae of the honeybee. The researchers discovered that the wasp diet was marginally suitable for larval development of *Chilocorus infernalis* Mulsant, *Chilocorus bipustulatus* (L.), and *Chilocorus cacti* (L.). Body weight of individuals reaching the adult stage

(e.g., *C. cacti*) was significantly less when the wasp diet rather than natural prey (scales) was the food source since eclosion. Adults fed artificial diets alone did not oviposit (Henderson et al., 1992).

Research also considered the potential of artificial diets to rear the vedalia beetle *R. cardinalis*, an important predator of scale insects (Matsuka et al., 1982). Adult females lived longer, but produced significantly fewer eggs, when fed an artificial diet of powdered honeybee brood with sucrose rather than live prey, *I. purchasi*. Interestingly, feeding adults sucrose for 2 days and *I. purchasi* for 1 day resulted in oviposition rates that exceeded that of the control group (i.e., adults fed *I. purchasi* alone). The authors stated that this feeding routine would permit continuous rearing of *R. cardinalis*.

Rearing of predatory beetles other than coccinellids has met with limited success. *Dastarcus helophoroides* (Fairmaire) is a colydiid beetle that attacks wood-boring insects, including cerambycid beetles and xylocopid bees. Ogura et al. (1999) evaluated artificial diets containing some insect components as food for this predator. Rearing newly hatched larvae on diets composed of silkworm pupa-powder, dry yeasts, yeast extract, sucrose, peptone, squid liver oil, preservatives, and distilled water, resulted in very low adult emergence rates (i.e., less than 10%). However, if larvae were fed with paralyzed cerambycid larvae until achieving a body length of approximately 8 mm, then reared on an artificial diet, adult emergence rates were much improved (i.e., greater than 50%). *D. helophoroides* larvae did not commonly cannibalize siblings even when reared in groups. Ogura and Hosoda (1995) tried to rear *Trogossita* (=*Temnochila*) *japonica* Reitter, a trogossitid predator of bark and wood-boring beetles, on artificial diets. A diet consisting of silkworm pupa-powder, dry yeasts, sucrose, peptone, squid liver oil, agar and distilled water supported the development of *T. japonica* larvae. Although diet-reared larvae developed into smaller adults, they mated and oviposited at normal levels when provided with natural prey (Ogura and Hosoda, 1995).

Artificial diets without arthropod components: Research has shown very limited success in rearing predators on artificial diets devoid of any arthropod components. Atallah and Newsom (1966) found that incorporating a filtered extract of cotton leaves (containing carotenoids and sterols) into an artificial diet based on protein from casein, soybean hydrolysate, and a liver fraction was satisfactory for rearing the pink-spotted lady beetle *C. maculata* for eight consecutive generations with low mortality after the second generation. Unfortunately, the authors did not provide control (e.g., natural, or factitious prey) to compare with the results of the artificial diet. The addition of vitamin E to the adult diet increased copulation of *C. maculata* adults. The observation that a diet that lacked the filtered extract of cotton leaves was not satisfactory for the development of this predator (Atallah and Newsom, 1966) suggests the presence of a stimulatory substance in cotton leaves that could possibly be isolated and incorporated into new and improved diets.

Experiments considered the potential of three formulations of a chicken liver-based artificial diet on the development, weight gain, and survival of *H. convergens* larvae (Hussein and Hagen, 1991). Results indicated that *H. convergens* larvae survived on a formulation containing powdered chicken liver, yeast hydrolysate, and sucrose at a ratio of 1:1:2. However, a natural diet of pea aphids, *A. pisum*, was best for optimum weight gain and development of this predator. The authors suggest that the growth and development of *H. convergens* larvae could improve by adding egg yolk to the diet formulation. Note that *H. convergens* adults are predators of nonaphid prey such as adults of the silverleaf whitefly *Bemisia argentifolii* Bellows & Perring and eggs of the pink bollworm *Pectinophora gossypiella* (Saunders) (Hagler, 2009).

Researchers evaluated the potential of three commercial liver extracts (i.e., labeled as S, L, and 2) in combination with an experimental oligidic diet, on the growth and development of *H. convergens* larvae (Racioppi et al., 1981). They compared the performance of their diet with a previously established Vanderzant diet. The time required to complete development was shorter and the body weight of adults was greater for individuals reared on the experimental diet (with or without liver extracts) in comparison to the Vanderzant diet. However, when fed live aphids (*S. graminum*), development time was shorter and the body weight (i.e., body mass) of pupae and adults was greater than cohorts fed the experimental diet containing the "S" liver extract. Removal of tryptophan and cystine from the diet resulted in deformed adults, as evidenced by underdeveloped tibia and tarsi.

A series of artificial diet formulations were developed and compared with natural prey as suitable food for rearing *A. bipunctata* (Kariluoto et al., 1976). The main ingredients in the diet formulations included variable amounts of wheat germ, Brewer's yeast, sucrose, honey, chicken eggs, casein, liver, a salt mixture, vitamins, antibiotics, agar, and water. Even when using the most effective formulation, development time was 20%−30% longer than that required by individuals fed natural prey (aphids, *M. persicae*). Adult emergence was 60%−80% versus 65%−95% when reared on the best formulation versus natural prey, respectively. Although, females did not oviposit when reared on the best diet formulations, some adults were able to live for 6 months.

Bonte et al. (2010) compared an artificial diet based on protein from beef and chicken supplemented with pollen with natural prey (pea aphids) on the life parameters of *A. bipunctata*. Their diet had negative effects on the life cycle

of this predator (Table 2.3), but adult longevity was unaffected. Experiments were conducted with two antibiotics, namely sorbic acid and methyl-4-hydroxybenzoate (MpHB) to retard the growth of mold (e.g., *Aspergillus niger*, *Penicillium* spp.) on an artificial diet used for rearing *A. bipunctata* (Kariluoto, 1978). Five concentrations (ranging from 0 to 2000 ppm) of both compounds were tested in 25 combinations. Sorbic acid was more effective than MpHB and a 1500-ppm concentration of sorbic acid controlled mold on the artificial diet for 3 weeks at 25°C. Unfortunately, an increase in the concentration of both compounds increased larval development. Adult emergence, however, was the highest (i.e., 76%) when a concentration of 1000 ppm of SA was used in the larval diet. No concentration of either antibiotic negatively affected adult weight.

Khan and Khan (2002) experimented with an artificial diet based on powdered chicken liver and sucrose to rear the coccinellid *Menochilus sexmaculatus* (F.), a predator of aphids, psyllids, and scale insects in Southeast Asia. Unfortunately, the chicken liver diet was inferior to natural prey, such as the green peach aphid *M. persicae* (Table 2.3).

Sighinolfi et al. (2008) compared the effects of a pork liver-based artificial diet to factitious prey (*E. kuehniella* eggs) on *H. axyridis* larvae and adults. Although complete development from first instar larva to mature adults was possible when using the artificial diet, differences in both food sources on predator life history parameters evidenced some nutritional deficiencies in the artificial diet (Table 2.3). Dong et al. (2001) tested four artificial diets for rearing *H. axyridis* in the laboratory, including the egg yolk diet, whole egg diet, gelatin diet, and starch diet. All four diets contained the following base ingredients (in equivalent quantities): chicken liver, cane sugar, honey, Brewers' yeast, casein enzymatic hydrolysate, soy oil, salt, and vitamins. The whole egg diet produced a survival rate of 82.5% in the first generation and three generations were tested. The starch diet produced a first-generation survival rate of 97.5% and two generations were tested. The gelatin diet produced the lowest survival rate. In comparison to factitious prey (i.e., eggs of the grain moth or the pink bollworm), the starch diet and the whole egg diet met similar expectations in terms of survival rate, development rate, adult body weight, and fecundity (Table 2.3).

Yazdani and Zarabi (2011) had limited success rearing *C. arcuatus*, a coccinellid that attacks whiteflies, on an artificial diet consisting of yeast, honey, and pollen (Table 2.3). When compared against live prey such as the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood), this artificial diet resulted in predators that were less fecund and fertile. However, adult males and females lived longer when fed the artificial diet rather than whiteflies (*T. vaporariorum*).

Research to develop artificial diets for predators of the hemlock woolly adelgid *Adelges tsugae* (Annand) is ongoing. Preliminary work by Cohen et al. (2008) demonstrates the possibility of developing an artificial diet, devoid of arthropod components, for the coccinellid *S. tsugae*, which is one of the most important predators of *A. tsugae*. *S. tsugae* will feed on a chicken egg-based artificial diet when hemlock woolly adelgids are in low supply or of inferior quality (Cohen et al., 2008). An artificial diet for the coccinellid would greatly expand the mass-rearing operations of this predator for release into forests in the northeastern USA.

Several researchers have attempted to rear noncoccinellids on artificial diets devoid of arthropod components. Weseloh (1998) tested an artificial diet for the carabid *Calosoma sycophanta* (L.), which is a predator of larvae and pupae of gypsy moth *Lymantria dispar* (L.) and other lepidopterans in forest ecosystems. Chicken meat and beef liver constituted the base protein in this diet. Larval development and survival were similar between the artificial diet and natural prey, *L. dispar* pupae. However, adults were smaller when reared as larvae on the artificial diet rather than natural prey.

Lundgren et al. (2005) developed a rearing protocol and observed aspects of the life history of a carabid *Poecilus chalcites* (Say) in the laboratory. *P. chalcites* is a common, generalist predator of ground-dwelling arthropods in several agroecosystems. Larvae completed development (with 80% pupation rates) when using a meridic diet based on commercially produced cat food. Note that the addition of other components to the diet (such as chicken egg or chicken liver) increased the body weight of *P. chalcites* larvae. Unfortunately, diet-reared adults failed to produce eggs.

An artificial diet based on veal, veal gravy, hen's eggs, potted meat, infant formula, and casein hydrolysate in Parafilm capsules was developed for the clerid, *Thanasimus dubius* (F.), an important predator of the scolytids *Dendroctonus frontalis* Zimmermann and *Ips grandicollis* (Eichhoff) (Reeve et al., 2003). The authors developed this diet to match the chemical composition of the tissues of *I. grandicollis*. Diet-reared individuals were similar in quality to those taken directly from the wild, regarding survival rate, longevity, and predation capacity. Diet-reared individuals maintained their capacity to kill natural or factitious prey even after five generations on the artificial diet. Costa and Reeve (2012) found that sorbic acid, a preservative to increase the shelf life of the diet, reduced the fecundity of *T. dubius* females by 20%−40% but had no measurable effect on adult longevity or body size. The authors concluded that sorbic acid was not an essential component in their diet, because removal of this preservative did not affect rearing efficiency.

Carcinops pumilio (Erichson) is a histerid predator of dipterans such as the housefly *M. domestica*. Research involved the testing of an artificial diet, containing a protein-rich food additive (PRO-PLEX™), versus natural prey (Achiano and Giliomee, 2006). Although predators could develop from egg to adult on the artificial diet, it took longer. Additionally, predators weighed less, the mortality rate was higher, and the oviposition rate was lower for individuals fed the artificial diet rather than natural prey (Achiano and Giliomee, 2006).

2.3 Rearing density and production

2.3.1 Crowding

Crowding is a behavioral response to changes in the density of populations under natural conditions in the field or artificial conditions indoors. Under artificial conditions in rearing cultures, crowding can have positive or negative consequences on insects in confined spaces. Peters and Barbosa (1972) reviewed the literature on density effects on the life history of insects in culture. They admitted that overcrowding was often detrimental to the development, body size, and fecundity of reared insects. Rearing density was inversely proportional to the survival of larvae of the histerid *C. pumilio* (Erichson), a predator of dipteran larvae (Achiano and Giliomee, 2004). An increase in *C. pumilio* larval density resulted in a decrease in larval survival in rearing containers. Omkar and Pathak (2009) found that crowding affected the coccinellid *Propylea dissecta* (Mulsant) immatures when reared in plastic beakers (11 × 9 cm, H × W). Rearing densities of 8, 16, 25, or 35 (rather than 4) larvae per beaker had negative effects on development time, larval survival, and adult emergence. Bista et al. (2012) considered the influence of crowding and diet (aphid species) on the development and survival of the coccinellid *Brumoides suturalis* (F.) in Petri dishes (14 × 1.5 cm, W × H). Rearing larvae of *B. suturalis* singly versus in groups (1 larva vs 10 larvae per dish) did not affect rearing success. However, prey species had a more significant impact; development was faster on *A. pisum* and larval survival greater on *Aphis gossypii* Glover (Bista et al., 2012). Riddick and Wu (2015a,b); demonstrated that *C. maculata* first instars could be reared in cohorts of up to 20 individuals in Petri dish arenas (159 cm^3, volume) with little or no negative effects on their development to pupae, then adults.

2.3.2 Cannibalism

Cannibalism is a normal behavioral event in the population dynamics of numerous animals living under natural conditions (Fox, 1975). Investigators have more often observed cannibalistic behavior in the laboratory in arthropod colonies confined in enclosures (Peters and Barbosa, 1972). With respect to predatory beetles, Banks (1956) showed that cannibalism was a significant source of mortality in the egg stage in coccinellids; emerging larvae consumed the unhatched eggs of their siblings, even at low population density. Common costs associated with larval cannibalism of eggs rather than feeding on moth (*Ephestia*) eggs were longer development time and reduced adult size in at least two coccinellids, *H. axyridis* and *Olla v-nigrum* (Mulsant) (Michaud, 2003). Adult coccinellids cannibalized their own eggs and unrelated eggs, especially when natural prey was in low supply (Cottrell, 2005). Cannibalism of eggs has beneficial effects on the nutrition of coccinellids. Dimetry (1974) found that *A. bipunctata* larvae could develop to the adult stage solely on a diet of conspecific eggs. *Adalia bipunctata* may even live longer when feeding on conspecific eggs rather than their prey (aphids), especially during periods of prey shortage (Agarwala, 1991). Egg cannibalism allowed larval development of the coccinellid *H. axyridis* on low-quality prey, such as nutrient deficient aphids (Snyder et al., 2000). The recognition of siblings (kin) may have some bearing on the occurrence of cannibalism. Agarwala and Dixon (1993) found that *A. bipunctata* adult females were reluctant to eat their own eggs, but adult males did not appear to avoid the eggs they sired, which suggests that adult females recognized their own offspring.

Despite the nutritional benefits of cannibalism and the capacity of some adults to recognize their own siblings, the propensity of coccinellids to cannibalize their siblings or offspring could hamper attempts at developing space-saving techniques for mass rearing coccinellids in support of augmentative biological control (Allen and Riddick, 2012). Assuredly, cannibalism is a key obstacle to the efficient mass production of coccinellids (Hodek, 1996). Researchers have attempted to curb cannibalism in laboratory arenas and cages over the years. For example, Shands et al. (1970) used excelsior (slender shavings of wood) to increase traveling space in the base of oviposition cages for *C. septempunctata* adults so that they would be less inclined to encounter and cannibalize eggs that had been oviposited on corrugated pasteboard.

Cannibalism is a common behavior of other predators such as carabids under confined conditions in the laboratory. Weseloh (1996) used moist peat moss (3 cm deep, at the base of plastic rearing containers) to reduce cannibalism

amongst larvae of the carabid *C. sycophanta* by 50%. Symondson (1994) did not observe cannibalism of eggs or larvae when rearing larvae of the carabid *Abax parallelepipedus* Piller & Mitterpacher in batches, in containers with earthworms as prey, and with peat and a small amount of clay as the substrate. This predator was a candidate biocontrol agent of slugs such as *Deroceras reticulatum* (Muller) in protected horticulture in the UK (Symondson, 1994). The cannibalistic behavior of some predators can be reduced by manipulating the rearing substrate and providing refugia to reduce contact between siblings and ensuring that food is of suitable quality and quantity.

2.3.3 Design of oviposition substrates and rearing enclosures

Natural host plants are typical oviposition substrates for coleopteran predators in the field (Vatansever et al., 2003). Natural and target prey are often reared on host plants, which provide oviposition sites for predators near their prey, in cages in the field or laboratory. For example, in rearing facilities in the eastern USA, researchers are producing the lady beetle *S. tsugae* on target prey (the hemlock woolly adelgid) on hemlock twigs inside cages (Conway et al., 2010), and adults oviposit on Curad gauze as often as hemlock twigs, at least when adult females are confined inside 3.8 L glass jars. This study demonstrated the feasibility of using nonplant material as oviposition substrates in lieu of host plants. Eliminating live plants from cages would reduce costs associated with rearing three trophic levels (host plants, prey, and predator). Eliminating plants could reduce the harmful effects of plant defenses on developing coccinellids. Research has shown that plant trichomes and plant toxins (allelochemicals) can reduce the growth and development of coccinellids (Riddick and Wu, 2010b; Riddick et al., 2011b).

Some alternative substrates suitable for oviposition include synthetic products. For example, the pink-spotted lady beetle *C. maculata* preferred ovipositing on textured surfaces (Kimwipes) rather than smooth surfaces (Parafilm paper backing) in laboratory cages (Allen and Riddick, 2012). The two-spot ladybird *A. bipunctata* laid more eggs per clutch on filter paper than on a natural substrate (spruce needles) in the laboratory (Timms and Leather, 2007). A coccinellid *Diomus terminatus* (Say), a predator of the corn leaf aphid, *Rhopalosiphum maidis* (Fitch), oviposited on wax paper strips and on sorghum leaves in laboratory containers (Tifft et al., 2006).

For some coccinellids, the application of a chemical cue from natural prey or host plants stimulates oviposition onto alternative substrates. Merlin et al. (1996a) found that chemical cues in the wax filaments of the mealybug *P. citri* stimulate oviposition in *C. montrouzieri*. Smirnoff (1958) stated that several mass-produced coccinellids did not oviposit in large cages unless a twig from the natural host plant was available. Extracts from *Juniperus virginiana* (L.) wood stimulated oviposition in *C. maculata* in the laboratory (Boldyrev et al., 1969). Ethanol extracts from cinnamon, clove, and teak also stimulated oviposition in *C. maculata* (Smith and Williams, 1976). Flavonoid compounds, such as quercetin and taxifolin, can stimulate oviposition by *C. maculata* in cages in the laboratory (Riddick et al., 2018a,b, 2019). Treatment of surfaces with extracts from fennel and cypress, in comparison to untreated surfaces, stimulated oviposition in *A. bipunctata* (Iperti and Prudent, 1986). In a preliminary study, Allen and Riddick (2012) found that methyl salicylate did not improve the oviposition of *C. maculata* inside cages. Methyl salicylate is a herbivore-induced plant volatile known to attract beneficial arthropods including the coccinellid *Stethorus punctum picipes* (Casey) in the field (James, 2005).

The physical presence of other predators (conspecific or heterospecific) or the odors that they leave behind, in feces or tracks, can deter oviposition (Seagraves, 2009). Chemical cues produced by conspecific larvae deter oviposition in *C. montrouzieri* (Merlin et al., 1996b). Little is known about the compounds that deter oviposition of coccinellids and other coleopteran predators in mass production systems. More research is needed to determine how to enhance oviposition while discouraging oviposition deterrence in a range of coccinellids within enclosures and to determine the effect of cage size on the efficacy of oviposition stimulants for coccinellids.

There is no standard enclosure (cage) design for mass rearing of coleopteran predators. The design of cages could have important implications for large-scale production and automation of the rearing process (Smith and Nordlund, 1999). Space is usually limited in many rearing facilities, so using small rather than large cages would seem logical. However, the biology of the species under rearing is critical for selecting an optimal cage size and shape. Cannibalism could become a problem as population density increases with decreasing or no change in cage size. Investigators have constructed rearing cages from various materials such as plastic, polypropylene, Plexiglas, glass, and wood. The only common factor among the different styles and materials used in designing these cages is the use of screens on one or more sides or the top (lid) of the cages. The screens are metal or aluminum wire, nylon, or muslin fabric of variable mesh sizes to prevent the escape of developing predators and prey while providing air circulation. The orientation of cages can also affect the production of coleopteran predators. A simple change from vertical to horizontal orientation of hemlock twigs and artificial oviposition substrates in 3.8 L glass jars increased egg production of the coccinellid *S. tsugae* (Conway et al., 2010).

2.3.4 Rearing scale

The ability to envision large-scale production of any natural enemy should be an integral part of the initial stages of planning a rearing program (Nordlund, 1998). To our knowledge, published research that attempts to translate rearing results for coccinellids or other coleopteran predators into a large-scale application is limited. Whistlecraft et al. (1985) developed a technique to rear the staphylinid *Aleochara bilineata* (Gyllenhal), a predator-parasitoid of the root maggots *Delia radicum* (L.) and *Delia antiqua* (Meigen). Adult beetles accepted second and third instar *D. antiqua* larvae as prey in plastic containers lined with a layer of moist sand under complete darkness at 14°C. Adults also oviposited in the sand at a rate of six eggs per female per day. First instar *A. bilineata* larvae searched for and parasitized maggot pupae at a relatively high rate (71%) when held in containers also lined with moist sand at 22°C and long photoperiod (L:D, 16:8), to prevent *A. bilineata* larvae from entering diapause. Whistlecraft et al. (1985) found that approximately 10,000 *A. bilineata* adults resulted from just 5 hours of human labor per week.

Lawson and Morgan (1992) described a method of rearing large numbers of a clerid beetle *T. dubius* and a trogossitid beetle *Temnochila virescens* F. using pine billets (0.8 m long, 22.5 cm wide) infested with the bark beetle *I. grandicollis*. At a harvest rate of 12 predator larvae per billet, with three billets per week, approximately 1850 mature larvae were produced per year. The authors achieved this level of production by maintaining the colonies at 25°C in an insectary rather than a laboratory.

Greene (1996) reared the predatory staphylinid *Creophilus maxillosus* L. on blowfly *Calliphora* spp. larvae in complete darkness in plastic boxes (11 cm^2, 3.5 cm deep) lined with damp sand. Females laid an average of 500 eggs/day at a temperature of 28°C in this system with a 30% survival rate from egg to adult stage, producing 150 adults. In theory, nearly 422,000 females (third generation) would result within approximately 90 days (Greene, 1996).

Although production of coccinellids with natural or target prey can be expensive, Pickett et al. (1999) developed a method of rearing *Delphastus catalinae* (Horn) in the laboratory using whiteflies *B. argentifolii* as prey. Using poinsettia as host plants, more than 174,000 *D. catalinae* adults were harvested in a 20-week time frame. They discovered that harvesting 50% of the adults per week stabilized the colony and maximized production. The cost of producing this beetle was US$0.22, which included mostly labor costs (Pickett et al., 1999).

Salom et al. (2012) described the sequence of developments that led to their current system for rearing the derodontid beetle *Laricobius nigrinus* Fender, another important predator of the adelgid *A. tsugae*. With careful consideration of environmental conditions inside the rearing cages, the authors eventually produced over 19,000 beetles/year. The availability of live prey was critical to the mass production of this predator. The authors thought it was necessary to introduce wild (field-collected) beetles into the colony each year.

2.4 Temperature and production

2.4.1 Optimizing temperature for rearing

Temperature is an important abiotic factor influencing population growth and fitness of predatory arthropods in nature and in confinement (Hallman and Denlinger, 1998; Stathas, 2000). Numerous studies attest to the importance of manipulating temperature (along with other factors such as photoperiod and humidity) for rearing predators under conditions that maximize growth and fitness (Schüder et al., 2004; Schneider, 2009). Exemplary studies demonstrate the usefulness of life table analyses in determining the optimal temperature (or optimal temperatures within a narrow range) to rear coccinellids that prey upon aphids, scales, and whiteflies (Canhilal et al., 2001; Mota et al., 2008; Jalali et al., 2009b). Atlihan and Chi (2008) found that *Scymnus subvillosus* (Goeze) had its highest net reproductive rate at 25°C rather than 20°C, 30°C, or 35°C under laboratory conditions. *S. subvillosus* is an aphidophagous predator in agroecosystems, especially orchards, in Turkey. Another aphidophagous coccinellid, *H. axyridis*, had its highest net reproductive rate and population doubling time at 15°C rather than 20°C or 25°C (Castro et al., 2011); nevertheless, the authors claimed that a temperature of 25°C was most suitable for rearing *H. axyridis*. The aphidophagous coccinellid *P. dissecta* had its highest net reproductive rate at 27°C rather than 25°C, 30°C, or 35°C under laboratory conditions in North India and the authors considered this temperature the optimal for mass rearing (Pervez and Omkar, 2004). The whitefly predator *Nephaspis oculatus* (Blatchley) displayed the best population growth when reared inside incubators at 26°C, rather than 20°C, 23°C, 29°C, 31°C, or 33°C (Ren et al., 2002). Kutuk and Yigit (2007) determined that the whitefly predator *D. catalinae* reproduced at its highest rate at a temperature of 25° rather than 30°C or alternating between 25°C and 35°C (on a 12 hours/12 hours light/dark cycle); the authors concluded that a range between 25°C and 30°C was optimum for mass-producing *D. catalinae*. In a study using the same predator (*D. catalinae*), Legaspi et al. (2008) found that

population growth was best at 22°C and 26°C rather than 30°C. Morales-Ramos and Rojas (2017) investigated temperature-dependent biological and demographic parameters of the coccinellid *C. maculata*. The optimum temperature for *C. maculata* population growth was 25°C rather than 24°C, 26°C, 27°C, or 28°C. The highest intrinsic rate of natural increase (r_m, 0.066) and lowest population doubling time (*DT*, 10.57 d) were observed at 25°C.

2.4.2 Reducing temperature for cold storage

Cold storage of natural enemies is one of the most important strategies used to facilitate the mass production and utilization of natural enemies for biological control (Glenister and Hoffman, 1998; Leopold, 1998). Short-term storage could help the producer balance the differences in costs between product supply and demand; long-term storage could reduce production costs if rearing occurred only during the growing seasons. The perceived advantages of cold storage are (1) prolongation of lifespan by reducing metabolic rate, (2) shipment over long distances without high mortality from starvation or desiccation, and (3) synchronization of natural enemy abundance with prey/host abundance (Riddick, 2001). In the context of this chapter, cold storage involves techniques that reduce the metabolic rate of coleopteran predators to prolong their lifespan. Some knowledge of the physiology of the predator during the winter season of the year (in temperate zones of the world) can provide clues to more efficient cold storage. Dormancy (diapause, quiescence) is a major strategy used by arthropods, including predatory and parasitic species, to survive during harsh winter conditions (Leather et al., 1993). Denlinger (2008) states that diapause merits more attention from the scientific community.

Researchers interested in the long-term cold stage have attempted to simulate the winter season in the laboratory for several coleopteran predators (Table 2.4). Adults of the two-spot ladybird, *A. bipunctata*, were field-collected in August-September and placed inside artificial hibernacula for cold storage during the winter months in the laboratory (Hamalainen, 1977). Survival of *A. bipunctata* was good, 70% or more of the individuals survived until March and 50% survived until May. Neel and Solomon (1985) removed *C. maculata* adults from winter aggregations between December and March, in two consecutive seasons, and placed them inside cloth or paper bags then cold-stored them in incubators in the laboratory. The authors periodically (every 2−3 weeks) removed the bags to mist the insects to prevent dehydration. Up to 69% of adults survived in cold storage, depending on the month taken from aggregations. Survival was greatest for those individuals removed from aggregations after January each year. Faulds (1988) described methods of storing a clerid beetle, *Thanasimus formicarius* (L.), released for control of bark beetles *Hylastes ater* (F.) and *Hylurgus ligniperda* (F.). Storage of *T. formicarius* adults was feasible when adults were fed prior to storage and removed periodically (at 3-month intervals) for feeding. Within-storage mortality was only 4% and storage had only negligible adverse effects on fecundity. Abdel-Salam and Abdel-Baky (2000) had some success at storing *Coccinella undecimpunctata* L. in the laboratory when adults consumed aphids prior to entering storage. Seo and Youn (2002) stored prewintering adults of the coccinellid *H. axyridis* without food for more than 120 days with a survival rate of 84%. Riddick (2010) found that 100% of parasite-free *H. axyridis* adults (males and females) survived under simulated winter conditions in a laboratory refrigerator for 4.5 months. Ruan et al. (2012) and Awad et al. (2013) subjected field-collected prewintering *H. axyridis* adults to long-term cold storage with good survival rates and reproduction after removal from storage (Table 2.4). Finally, Whistlecraft et al. (1985) found that the staphylinid *A. bilineata* could be stored as diapausing larvae for up to 6 months with no appreciable reduction in survival rate.

In attempts to facilitate short-term cold storage of coleopteran predators, researchers have subjected life stages that do not typically undergo winter conditions at low temperatures (see Table 2.4). Hamalainen and Markkula (1977) found that eggs of *A. bipunctata were* stored for 2 weeks had a survival rate of 55%−65% when returned to room temperature. The hatch rate of nonstored *A. bipunctata* eggs was approximately 68%. *C. septempunctata* eggs could be stored for just 1 week at a hatch rate of 65%, when returned to room temperature. The hatch rate of nonstored *C. septempunctata* eggs was 75%−80% (Hamalainen and Markkula, 1977). Abdel-Salam and Abdel-Baky (2000) determined the potential of storing egg, larval, or pupal stages of the coccinellid *C. undecimpunctata* in the laboratory. They discovered that eggs could be effectively stored at this temperature for up to 1 week, with a hatch rate of 65%; no eggs hatched after 2 weeks of storage. Larvae and pupae could safely be stored for 2 weeks and 1 week, respectively, without significant declines in larval survival and adult emergence from pupae.

The potential of storing larval stages of *C. maculata lengi* Timberlake was assessed in the laboratory by Gagné and Coderre (2001) using insects cultured for more than 10 consecutive generations on a diet of beef liver and moth (*E. kuehniella*) eggs. They found that second and third instars stored successfully for 2 weeks, without considerable adverse effects on the development and predation potential (i.e., voracity) of larvae when returned to rearing conditions. Miller (1995) showed that eggs of the coccinellid *E. connexa* could be stored for 1 day without negative effects on hatch rate; pupae, on the other hand, were stored at the same temperature for 3 weeks with no appreciable mortality.

TABLE 2.4 Coccinellid predators[a] and effects of cold storage on survival rate.

Predatory beetle	Storage conditions	Results	References
Adalia bipunctata (adults)	6°C, 70%–90% relative humidity (RH)	70% alive, 6 months	Hamalainen (1977)
A. bipunctata (eggs)	10°C, 80%–90% RH	55%–65% hatched, 2 weeks	Hamalainen and Markkula (1977)
Coccinella septempunctata (eggs)	10°C, 80%–90% RH	65% hatched, 1 week	Hamalainen and Markkula (1977)
Coccinella undecimpunctata (adults)	6°C, 60% RH	50% alive, 1.5 months, with feeding	Abdel-Salam and Abdel-Baky (2000)
C. undecimpunctata (eggs)	6°C, 60% RH	65% hatched, 1 week	Abdel-Salam and Abdel-Baky (2000)
C. undecimpunctata (larvae)	6°C, 60% RH	15% alive, 2 weeks	Abdel-Salam and Abdel-Baky (2000)
C. undecimpunctata (pupae)	6°C, 60% RH	65% alive, 2 weeks	Abdel-Salam and Abdel-Baky (2000)
Coleomegilla maculata (adults)	4.4°C, 60%–70% RH	69% alive, 6 months	Neel and Solomon (1985)
C. maculata (larvae)	8°C, 60%–70% RH	90%–100% alive, 2 weeks; predation (✓)	Gagné and Coderre (2001)
Eriopis connexa (eggs)	4°C, RH not reported	86% alive, 1 day	Miller (1995)
E. connexa (pupae)	4°C, RH not reported	100% alive, 3 weeks	Miller (1995)
Harmonia axyridis (adults)	4° and 8°C, RH not reported	84% alive, 4 months	Seo and Youn (2002)
H. axyridis (adults, parasite-free)	8°C, 60% RH	100% alive, 4.5 months	Riddick (2010)
H. axyridis (adults)	3° and 6°C; 60%–70% RH	80% alive, 5 months; fecundity (✓)	Ruan et al. (2012)

Symbol (✓) indicates no significant effect on life parameter.
[a]*Data on noncoccinellid beetles included in text only.*

Much more research is necessary on the response of coleopteran predators to low temperature in relation to production. Recent research has indicated that *Hippodamia variegata* (Goeze) adults could be stored for 35 day at 6°C, with a survival rate of 90%, without reducing female reproductive capacity (i.e., fecundity) when returned to rearing conditions in the laboratory (Sakaki et al., 2019). *Rhyzobius lophanthae* (Blaisdell) adults were stored for 20 day at 4°C, with a survival rate of 58%, and females produced an average total of 738.30 eggs poststorage (Şenal et al., 2017).

Strategies that could enhance the potential to cold store predators involve the use of cryoprotectant and carbohydrate molecules (Riddick and Wu, 2010a) and other molecules that protect the cells and tissues of predators during harsh conditions in the field. Storage of some insect eggs (such as heteropteran and lepidopteran) destined to serve as artificial hosts for parasitoids was possible in liquid nitrogen (Leopold, 1998). To our knowledge, no coleopteran predator or any other natural enemy has been stored successfully in liquid nitrogen. Diapause induction is another technique that holds promise in enhancing the storage potential of predators (Chang et al., 1996). Researchers could also explore the usefulness of inducing coleopteran predators to transition into a state of diapause.

2.5 Quality control and production

2.5.1 Safeguards against unwanted pathogens and parasites

Coccinellids and other coleopteran predators have the potential to harbor a diversity of parasites and pathogens under natural conditions in the field and artificial conditions in the laboratory (Ceryngier and Hodek, 1996; Bjørnson and

Schütte, 2003; Riddick et al., 2009; Riddick, 2010; Ceryngier et al., 2012). These pathogens can hinder the development of immature stages and lead to the demise of some individuals. Parasites and pathogens could inhibit the reproductive performance of adults. They could undermine biocontrol efforts as well as lead to the inadvertent introduction of parasites and pathogens into new locations. Bjørnson (2008a) found that *H. convergens* adults, shipped from three companies, destined for field releases, harbored several pathogens including microsporidia, eugregarines, and a braconid endoparasitoid. To prevent the inadvertent spread of pathogens from one locality to the next, workers should screen the coccinellids prior to field releases.

2.5.2 Preventing colony deterioration

Continuous mating between closely related individuals (i.e., inbreeding) in a population of insects over many generations can have detrimental effects on the health of a colony and on the performance of individuals released in the field (Mackauer, 1976; Roush and Hopper, 1995). Inbreeding may reduce the fitness of coccinellids such as *A. bipunctata*, *C. maculata*, *H. axyridis*, and *Propylea quatuordecimpunctata* (L.) (Kidd, 1993; Hurst et al., 1996; Morjan et al., 1999; Facon et al., 2011; Seko et al., 2012). Methods to reduce or prevent the deterioration of colonies may include manipulative breeding between hybrids to restore normal growth, survival, and reproductive capacities of populations in culture. Mating between siblings should be avoided by mixing adults with different parenthood origins. The practice of periodically adding wild (field) adults into well-established laboratory cultures to mate with domesticated adults could prevent inbreeding depression and subsequent reduction in fitness. Continuous monitoring of colonies for any undesirable changes in the behavior of adults, in comparison to adults found in the field, is necessary (Boller, 1972; Huettel, 1976).

2.5.3 In-shipment, postshipment and prerelease assessments

In-shipment effect of shipping containers on beneficial arthropods has received limited attention to date (Riddick and Morales-Ramos, 2017). A study by Bjørnson and Raworth (2005) showed that Styrofoam boxes do not adequately maintain internal temperatures. Consequently, beneficials are often subject to external temperatures during transport from commercial companies and release site. The authors suggest refrigerating the Styrofoam boxes (with beneficials inside) during shipment.

The sex ratio of individual species purchased from commercial companies can represent a measure of quality in a mass-production system. Heimpel and Lundgren (2000) purchased predators (coccinellids) and parasitoids from insectaries and used log-likelihood goodness-of-fit tests (Sokal and Rohlf, 1981) to determine if the sex ratio of adults of each species differed from a proportion of 0.5 males. Of the six species of coccinellids (*C. montrouzieri*, *D. pusillus*, *H. axyridis*, *H. convergens*, *Rhyzobius* (*Lindorus*) *lophanthae* (Blaisdell), *S. punctillum*), none deviated significantly from the expected sex ratio. O'Neil et al. (1998) assessed the postshipment quality of the convergent lady beetle, *H. convergens*, from several commercial companies. The percentage of adults alive upon arrival at their laboratory ranged from 50% to 81%, with no significant differences between companies. The percentage of unparasitized adults was 78% to 91% and females represented from 58% to 68% of the shipments, without any significant differences between companies. Finally, 67% to 80% of females produced eggs after arrival in the laboratory, irrespective of the company. Bjørnson (2008b) found that *H. convergens* adults, shipped from three commercial companies, did not commence oviposition until approximately 5 days later when fed aphids in experimental arenas in the laboratory.

Researchers have shown that the ability of mass-produced insects to take flight (i.e., flight capacity) is a potentially useful measure of the quality of a beneficial insect prior to its release (Couillien and Gregoire, 1994; van Lenteren, 2003). The longer that the rhizophagid beetle *Rhizophagus grandis* Gyllenhal remained in cold storage (at 3°C−7°C), the less likely adults could take flight in a wind tunnel (Couillien and Gregoire, 1994). Thus, prolonged cold storage beyond several months may have negative consequences on the ability of *R. grandis* to disperse from release sites in search of prey (bark beetles). The capacity to fly is not always a useful measure of quality. Individuals of a nonflying strain rather than the normal (wild type) strain of the coccinellid *H. axyridis* remained and oviposited on plants in greenhouses for a longer time (Ferran et al., 1997); fitness did not differ between wild type and nonflying strains (Tourniaire et al., 2000).

Quality control guidelines are established or currently being developed for a range of natural enemies (van Lenteren et al., 2003). These guidelines often include prerelease assessments of the performance of a natural enemy in a laboratory setting. Thus far, provisional quality control guidelines are available for just one predatory beetle, namely *C. montrouzieri* (van Lenteren et al., 2003).

2.6 Conclusions and recommendations

2.6.1 Synthesis

In this chapter, research related to producing coleopteran predators primarily for augmentative biological control has been highlighted. Rearing coleopteran predators on natural prey reared on host plants, is the mainstay in production research to date. Some alternative prey/foods such as lepidopteran moth eggs and brine shrimp eggs are stand-alone factitious foods for a few coccinellids and supplements in artificial diets for several others. Plant-based products such as honey, pollen, and royal jelly are supplements to or additives in some artificial diets and represent beneficial sources of nutrients. Novel methods that incorporate feeding stimulants into diets to incite feeding on nonprey or artificial diets by specialists need further exploration. Most of the artificial diets based on vertebrate protein, with or without arthropod components, are not as effective as natural prey or factitious food for production on coleopteran predators.

The capacity to rear coleopteran predators within the confines of a laboratory cage is amazing, given that many species travel considerable distances in search of food, shelter, and mates in nature. Knowledge on how to design cages and establish rearing densities within the confines of these cages to encourage and stimulate feeding, mating, and oviposition, while discouraging cannibalism, is increasing. The ability to manipulate temperature to maximize growth and development or to decrease metabolic rates for stockpiling or for overwintering is possible for several species. Several studies point out the necessity to monitor colonies for unwanted pathogens, and parasites as well as to ensure that coleopteran predators of high quality are being produced for the biocontrol market. Pre- and postshipment assessments of the health of products are important to the sustainability of any production system.

2.6.2 Future research

- The current criteria to measure the success or failure of factitious prey or an artificial diet for coleopteran predators may need modification. These criteria typically include the following life parameters: development time, larval survival, adult emergence, body size, oviposition (fecundity), and adult longevity. Previous research has shown that most artificial diets created thus far have less than ideal effects on these life parameters when compared to what is possible using natural or target prey. By far, one of the most important life parameters, which is often overlooked, is predation potential (Grenier and De Clercq, 2003). After rearing on factitious prey or an artificial diet for multiple generations, the capacity of a coleopteran predator to locate, capture, kill, and then consume target prey upon initial contact, after minimal conditioning before release, is a rational estimate of success.
- There is evidence that generalist (polyphagous) coleopteran predators can undergo adaptation to specific prey after several generations of exposure, at least in the laboratory (Rana et al., 2002). Adaptation to new prey also occurs in specialist (oligophagous) coccinellids (Ragab, 1995; Causton et al., 2004) and carabids (Weber et al., 2006; Weber and Riddick, 2011). More research is necessary to predict which species are most likely to adapt to an arthropod-free artificial diet.
- Detailed research is necessary to determine how to manipulate rearing (population) densities, relative to food quality/quantity, cage size, oviposition, and mating, to reduce the negative effects of crowding and cannibalism in colonies. Although cannibalism can have benefits on the health of an individual, excessive levels of cannibalism are often detrimental to overall colony health.
- More research is needed to establish rigorous measures of "quality control" (i.e., regular monitoring of products, before or after shipment to customers, for acceptable fitness and unwanted symbionts) for more coleopteran predators. Then specific standards can be developed to allow producers to assess the fitness of their products prior to sale.
- Development of automated systems for mass rearing is critical to long-term utilization of natural enemies in augmentative biological control. Utilization of machinery to reduce manual labor in the rearing process might increase cost-effectiveness. Smith and Nordlund (1999) claim that automation, that is, replacement of human labor with mechanical and/or electrical components, would lower costs, increase production, and improve quality of reared insects. Scaling-up the rearing operation to produce millions of predators is necessary (Nordlund, 1998; Smith and Nordlund, 2000).

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Chapter 3

Production of heteropteran predators

Patrick De Clercq[1], **Thomas A. Coudron**[2] **and Eric W. Riddick**[3]

[1]*Department of Plants and Crops, Ghent University, Ghent, Belgium,* [2]*Biological Control of Insects Research Laboratory, USDA-Agricultural Research Service, Columbia, MO, United States,* [3]*Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States*

3.1 Introduction

True bugs (order Hemiptera, suborder Heteroptera) are characterized by piercing-sucking mouthparts with which they pierce plants or animals to suck up their fluids or liquefied tissues. Feeding mechanisms of phytophagous and carnivorous bugs have been described in more detail by Hori (2000) and Cohen (2000b), respectively. In short, predatory bugs use a feeding method termed "solid-to-liquid" feeding: they inject digestive enzymes into the prey's body and then suck up the digested and liquefied tissues (Cohen, 1990, 2000b). This extra-oral digestion allows them to utilize the high nutrient prey tissues besides hemolymph and predisposes them to attack relatively large prey (Cohen, 2000b). In addition, many predatory heteropterans inject a venom through their mouthparts that quickly immobilizes prey.

It has been estimated that 65% of all families within the Heteroptera are partially or entirely composed of carnivorous species (Henry and Froeschner, 1988; Cohen, 2000b), and thus many of these may have potential for use in biological control of arthropod pests. In fact, several species of predatory bugs are economically important biological control agents of key agricultural pests. Species from the families Anthocoridae, Miridae, Geocoridae, Nabidae, Reduviidae and Pentatomidae (subfamily Asopinae) have been or are being used in augmentative biological control programs in various agroecosystems (Table 3.1).

Most of the carnivorous heteropterans used for biological control are polyphagous predators feeding on a wide array of arthropod prey. Flower bugs (Anthocoridae) of the genera *Orius* and *Anthocoris* have primarily been produced for the control of thrips and psyllids, respectively, but they also feed on a range of other small arthropods, including aphids. *Macrolophus, Dicyphus*, and *Nesidiocoris* spp. Are plant bugs (Miridae) feeding on whiteflies, but they also attack thrips, mites, aphids and other arthropod pests. Damsel bugs (Nabidae) of the genus *Nabis* have been evaluated against lepidopteran pests in various field and greenhouse crops, but their predation on aphids, psyllids, leaf hoppers and other insect pests has also received attention. The big-eyed bugs (Geocoridae) of the genus *Geocoris* are generalist predators feeding on whiteflies, thrips, mites, aphids, and eggs and small larvae of lepidopterans. Members of the stinkbug subfamily Asopinae are all predators feeding primarily on larvae of leaf-feeding lepidopterans, coleopterans and hymenopterans; economically important species are found in the genera *Podisus, Picromerus*, and *Perillus*. Finally, several assassin bugs (Reduviidae), including *Rhynocoris, Pristhesancus*, and *Zelus* spp., have been identified to be predators of key pests in diverse crop systems, but very few have been actively used in augmentation programs. More details on the biology and biological control potential of the main families and species of predatory Heteroptera can be found in Schaefer and Panizzi (2000).

According to van Lenteren (2011), the Palearctic species *Macrolophus pygmaeus* (Rambur) and *Orius laevigatus* (Fieber) are among the 12 economically most important invertebrate biological control agents, based on the number of countries in which the species are marketed. The commercial use of both species did not start before the early 1990s. It is worth noting that the former species was distributed commercially for some time in Europe under the erroneous identity of *Macrolophus caliginosus* Wagner, which is a different species [in fact, the presently accepted name of that species is *Macrolophus melanotoma* (Costa)]. This was most probably due to a misidentification based on unreliable morphological characteristics (Martinez-Cascales et al., 2006a,b; Machtelinckx et al., 2009). The species can only be reliably discriminated using molecular tools. As a result, several studies using insects purchased from commercial sources under the name "*Macrolophus caliginosus*" may in fact have studied *M. pygmaeus*.

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TABLE 3.1 Main species of predatory Heteroptera used in augmentative biological control programs, with indication of the primary target pests and the area where they have been or are currently used.

Species	Family	Main targets[a]	Area[b]
Anthocoris nemoralis	Anthocoridae	Psyllids	Europe, North America
Anthocoris nemorum	Anthocoridae	Psyllids, thrips	Europe
Orius albidipennis	Anthocoridae	Thrips	Europe, Africa
Orius insidiosus	Anthocoridae	Thrips	North and Latin America, Europe
Orius laevigatus	Anthocoridae	Thrips	Europe, North Africa, Asia
Orius majusculus	Anthocoridae	Thrips	Europe
Orius sauteri	Anthocoridae	Thrips	Asia
Orius strigicollis	Anthocoridae	Thrips	Asia
Orius tristicolor	Anthocoridae	Thrips	North America, Europe
Xylocoris flavipes	Anthocoridae	Stored product pests	North America, Africa, Asia
Nabis americoferus, Nabis roseipennis	Nabidae	Lepidopterans	North America
Nabis pseudoferus	Nabidae	Lepidopterans	Europe
Dicyphus hesperus	Miridae	Whiteflies	North America, Europe
Dicyphus tamaninii	Miridae	Whiteflies	Europe
Macrolophus melanotoma (=M. caliginosus)	Miridae	Whiteflies	Europe
Macrolophus pygmaeus	Miridae	Whiteflies	Europe, Africa
Nesidiocoris tenuis	Miridae	Whiteflies, lepidopterans	Europe, North Africa, Asia
Brontocoris tabidus	Pentatomidae	Lepidopteran defoliators	Latin America
Picromerus bidens	Pentatomidae	Lepidopteran and coleopteran defoliators	Europe
Perillus bioculatus	Pentatomidae	Colorado potato beetle	North America, Europe
Podisus maculiventris	Pentatomidae	Lepidopteran and coleopteran defoliators	North America, Europe, Asia
Podisus nigrispinus	Pentatomidae	Lepidopteran defoliators	Latin America
Geocoris punctipes	Geocoridae	Whiteflies, thrips, lepidopterans	North America
Rhynocoris marginatus	Reduviidae	Lepidopterans, heteropterans	Asia
Pristhesancus plagipennis	Reduviidae	Lepidopterans, heteropterans	Australia

[a]*Primary target pests are listed, although additional targets may occur.*
[b]*Both commercial and experimental releases are included.*
Source: Based in part on van Lenteren, J.C., 2011. The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake. BioControl 57, 1-20.

Several heteropteran families display trophic omnivory (also called zoophytophagy), allowing them to exploit both plant and prey resources (Coll, 1998; Coll and Guershon, 2002; Cohen, 1990, 2004; Torres and Boyd, 2009). Plants may be used to varying degrees as a source of water or supplementary nutrients. The acquired water may be essential for the process of prey feeding (Gillespie and McGregor, 2000). Their mixed feeding strategy may further allow them to perform better in terms of development and reproduction or survive periods of prey scarcity. Although in many cases zoophytophagous bugs do not damage plant tissues when piercing plant tissues, several predatory mirids such as *Nesidiocoris tenuis* (Reuter), *Dicyphus tamaninii* Wagner and *M. caliginosus* do damage plants (Sampson and Jacobson, 1999; Wheeler, 2001; Coll and Guershon, 2002; Calvo et al., 2009; Sanchez, 2009; Arnó et al., 2010), or

transfer plant pathogens (Burgess et al., 1983). Further, the adult females of predatory bugs may require plant tissue for egg deposition either on (e.g., Pentatomidae, Geocoridae, Reduviidae) or within (e.g., Anthocoridae, Miridae, Nabidae) the plant tissue. Their omnivory and need for plants as oviposition substrates may not only have implications for the practical use of predatory bugs in the field, but also has bearing on their mass production. In the present chapter, we set out to investigate how the zoophytophagous feeding habit of heteropteran predators contributes to the complexity of their mass production.

We will first provide an overview of natural, factitious and artificial foods that have been used for production of heteropteran predators. The use of plants and plant materials as sources of water and supplementary nutrients, and as living and oviposition substrates will be addressed, as well as the potential of alternative substrates. The impact of crowding and cannibalism and of the presence of microorganisms on the performance of rearing systems also will be addressed. Further, rearing systems for a selection of economically important species are described, with special attention to housing and rearing densities, and aspects of production scale. Future challenges and recommendations for research are presented in a concluding section.

3.2 Foods

3.2.1 Natural prey

Natural rearing systems for predators use the natural or target prey. This is often an herbivorous species maintained on plants or plant materials or, in some cases, reared on artificial diets (e.g., lepidopteran larvae). Natural rearing systems are essentially tritrophic, that is, they comprise three trophic levels: the predator, the herbivorous prey, and the prey's host plant. Although such systems have shown to be effective for the mass production of certain arthropod natural enemies, they may face problems of discontinuity and usually involve high costs related to labor and facilities for plant production (e.g., greenhouses) (Etzel and Legner, 1999; De Clercq, 2008; Riddick, 2009). Several studies have reported the use of natural prey for sustaining small scale cultures of heteropteran predators in research laboratories, but for the reasons mentioned above this type of prey is rarely used in mass production systems. Natural prey is often used as a benchmark to evaluate the nutritional value of unnatural or artificial foods. However, as many predatory heteropterans are generalist feeders there may be large differences in the nutritional quality of prey that is attacked by a given predator in its natural habitat. Therefore, the use of natural prey may not always result in the production of superior predators. In this context, the key to success in the rearing of predatory insects is to provide variation in the food offered, even when rearing on natural foods (Richman and Whitcomb, 1978; Evans et al., 1999; Lundgren, 2011).

Predatory stinkbugs of the genus *Podisus* are highly polyphagous and are relatively easily reared on larval forms of various insect prey. Although in some cases larvae of hymenopterans or coleopterans have been used for laboratory culturing, *Podisus* bugs have primarily been produced on lepidopteran larvae (De Clercq, 2000). Nymphs and adults of *Podisus maculiventris* (Say) and *Podisus nigrispinus* (Dallas) have been cultured on live caterpillars of several noctuids, including *Spodoptera* spp. (Yu, 1987; De Clercq et al., 1988; De Clercq and Degheele, 1993c), *Pseudoplusia includens* (Walker) (Orr et al., 1986), *Trichoplusia ni* (Hübner) (Biever and Chauvin, 1992), *Heliothis virescens* (F.) (Pfannenstiel et al., 1995), *Helicoverpa zea* (Boddie) (Warren and Wallis, 1971), *Alabama argillacea* (Hübner) and *Anticarsia gemmatalis* (Hübner) (Lemos et al., 2003; Torres et al., 2006). Other predatory pentatomids were reared on noctuid larvae, including *Eocanthecona furcellata* (Wolff) (Yasuda and Wakamura, 1992) and *Picromerus bidens* L. (Mayné and Breny, 1948; Mahdian et al., 2006). Most of these noctuid caterpillars can be reared on artificial diets devoid of living plant material, precluding the necessity for space for production of host plants. Peluzio et al. (2018), however, reported that *P. nigrispinus* performed better on *A. gemmatalis* caterpillars offered soybean leaves than on those fed on an artificial diet. Occasionally, caterpillars from other families have been used as food for rearing predatory pentatomids, like *Hyphantria cunea* (Drury) and *Malacosoma americanum* (F.) (Warren and Wallis, 1971). Frozen caterpillars proved to be a suitable diet for several predatory stinkbugs (Warren and Wallis, 1971; De Clercq et al., 1988; Sipayung et al., 1992; Yasuda and Wakamura, 1992); deep freezing surpluses from caterpillar cultures may thus allow for some flexibility in production systems using natural prey.

The two-spotted stinkbug, *Perillus bioculatus* (F.), is more restricted in its diet, although it reportedly attacks insects from various orders, including Lepidoptera, which is usually associated with chrysomelid prey in the field (see De Clercq, 2000 and references therein). Several workers reared *P. bioculatus* on eggs and early instar larvae of its target prey, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (e.g., Franz and Szmidt, 1960; Tremblay, 1967; Tamaki and Butt, 1978; Heimpel and Hough-Goldstein, 1994; Adams, 2000a), but this requires the continuous availability of potato foliage as no adequate artificial diet is available for the prey. Biever and Chauvin (1992), Coudron and Kim (2004),

and De Clercq (personal observation) found, however, that consecutive generations of *P. bioculatus* can be successfully reared on coddled larvae of *T. ni* or *Spodoptera littoralis* (Boisduval). Adams (2000a,b) used frozen larvae of *H. virescens* to rear the two-spotted stinkbug. The use of these noctuid prey renders production more cost-effective given the relative ease of rearing lepidopterans on artificial diets. Yocum and Evenson (2002); however, pointed out that for optimal results a diet of caterpillars had to be supplemented with eggs of the Colorado potato beetle, particularly for the early instars of the predator.

Noctuid prey was also found suitable for rearing predatory bugs from other families. The big-eyed bug, *Geocoris punctipes* (Say), was fed on coddled larvae of *Spodoptera exigua* (Hübner) by Champlain and Sholdt (1966,1967) and on eggs of *H. zea* or *Lygus hesperus* (Knight) by Cohen and Debolt (1983). Cohen and Urias (1986) reported superior results on a combination of heat-killed *S. exigua* larvae and *H. virescens* eggs. The damsel bugs *Nabis americoferus* (Carayon), *Nabis roseipennis* Reuter and *Nabis rufusculus* Reuter were maintained in the laboratory on fresh *H. virescens* eggs (Braman and Yeargan, 1988). Kiman and Yeargan (1985) used frozen eggs of the latter noctuid to maintain their colony of the anthocorid *Orius insidiosus* (Say). Nadgauda and Pitre (1987), on the other hand, reported that feeding *N. roseipennis* on larger *H. virescens* larvae effectively sustained the predator's development. Ables (1978) reared the assassin bug *Zelus renardii* (Kolenati) on *H. virescens* larvae, whereas Grundy et al. (2000) provided *Pristhesancus plagipennis* Walker with larvae of *Helicoverpa armigera* (Hübner). *Rhynocoris marginatus* (F.) and other reduviids were successfully reared on larvae of the target prey *Spodoptera litura* (F.) in India (Venkatesan et al., 1997; Sahayaraj and Paulraj, 2001).

Occasionally, other natural prey types also have been used to feed stock colonies of predatory bugs. For instance, aphid prey, including pea aphid, *Acyrthosiphon pisum* (Harris), and green peach aphid, *Myzus persicae* (Sulzer), have been used for culturing various predatory bugs like nabids (Guppy, 1986) and anthocorids (Ruth and Dwumfour, 1989). Yokoyama (1980) presented a rearing method for *Geocoris pallens* Stål using eggs and nymphs of milkweed bug, *Oncopeltus fasciatus* (Dallas), as food.

The warehouse pirate bugs *Xylocoris* spp. can be reared on various insects found in stored product habitats, where these generalist predatory bugs are naturally found. Chu (1969) was able to mass rear *Xylocoris galactinus* (Fieber) on larvae of *Tribolium castaneum* (Herbst) infesting cracked corn. Dunkel and Jaronski (2003) used similar methods to maintain a stock culture of *Xylocoris flavipes* (Reuter) on larvae of *T. castaneum* fed on wheat flour and brewer's yeast. Arbogast et al. (1971) showed that *X. flavipes* could be reared on eggs and first instars of *Plodia interpunctella* (Hübner). Press et al. (1973) later demonstrated that *X. flavipes* can be reared on *P. interpunctella* eggs that were killed by gamma irradiation or freezing, inspiring others to use eggs of lepidopteran storage pests as factitious prey for various predatory bugs.

3.2.2 Factitious prey

Costs of production may be reduced when predatory insects can be produced on unnatural or factitious prey that is easier and less expensive to rear than natural prey. Factitious prey is organisms that are not normally attacked by the predator, mostly because they do not occur in their natural habitat but can support their development in a laboratory environment (De Clercq, 2008). Factitious prey may be offered fresh, but in many cases, they are frozen, irradiated or lyophilized for improved storage or use in predator cultures (Riddick, 2009). Given that many heteropteran predators used in augmentative biological control programs are highly polyphagous, they are usually amenable to rearing on factitious prey (Riddick, 2009).

Lepidopteran eggs, particularly of the Mediterranean flour moth *Ephestia (Anagasta) kuehniella* Zeller, the Indian meal moth *P. interpunctella*, the rice moth *Corcyra cephalonica* (Stainton) (all Pyralidae) and the Angoumois grain moth *Sitotroga cerealella* (Olivier) (Gelechiidae) have been extensively used as a factitious food for various insect predators, including predatory bugs. Lopez et al. (1987) reported that frozen eggs of *S. cerealella* were suitable to temporarily support the rearing of the lygaeid *G. punctipes*, although they were considered inferior to fresh *Heliothis* eggs. Alauzet et al. (1992) and Blümel (1996) showed that the anthocorid *Orius majusculus* (Reuter) could be reared successfully on eggs of *E. kuehniella*. Fauvel et al. (1987) and Cocuzza et al. (1997) and Toft et al. (2020) found that the mirid *M. caliginosus* and the anthocorids *O. laevigatus* and *O. majusculus*, respectively, performed even better on *E. kuehniella* eggs than on some of its natural prey. Zhou and Wang (1989) reported similar findings for *Orius sauteri* (Poppius) fed on rice moth eggs. Since those early studies in the 1980s and 1990s, lepidopteran eggs, and in particular eggs of *E. kuehniella*, have been deemed a suitable food for the mass production of a wide range of heteropteran predators including *Orius* spp. (Fig. 3.1) (Richards and Schmidt, 1995; van den Meiracker, 1999; Tommasini et al., 2004; Kakimoto et al., 2005; Bueno et al., 2006; Bonte and De Clercq, 2008; Venkatesan et al., 2008; Sobhy et al., 2010; Ballal et al., 2012; Tuan et al., 2016; Bonte et al., 2017), *Geocoris* spp. (Oida and Kadono, 2012; Varshney and Ballal, 2017; Toft et al., 2020) and several

FIGURE 3.1 Adult of *Orius naivashae* feeding on *Ephestia kuehniella* eggs. *Photo: J. Bonte.*

Miridae spp. (Fauvel et al., 1987; Castañé et al., 2006; Calvo et al., 2009; Sanchez et al., 2009; Bueno et al., 2018; Varshney et al., 2019). Eggs of *E. kuehniella* were also suitable to sustain the development of the predatory pentatomids *P. maculiventris* and *P. bidens*, but the latter species could not produce eggs on this food (Mahdian et al., 2006). To avoid hatching of lepidopteran eggs in predator cultures, they are usually killed by deep freezing, or by gamma- or UV-irradiation (Etzel and Legner, 1999). In addition to their use in laboratory cultures, eggs of *E. kuehniella* (and probably those of some other lepidopterans) can be used to support populations of certain predatory bugs in field settings (Lenfant et al., 2000; Urbaneja-Bernat et al., 2013).

The continuous use of lepidopteran eggs as a factitious food in mass-rearing systems does have some drawbacks, the most important of which is their high cost. Although the moths are easily produced on inexpensive foods (flour or grains of cereals), there are substantial monetary investments for the mechanization of rearing procedures (handling of the rearing medium, harvesting the moths, harvesting and cleaning the eggs), climate management, and the health of workers (repeated inhalation exposure to scales is known to cause allergies). This has led to high market prices, especially for *E. kuehniella* eggs, which are approximately US$500/kg (De Clercq, 2008; K. Bolckmans and P. Couwels, personal communication). Interestingly, Urbaneja-Bernat et al. (2013) reported that supplementing a diet of *E. kuehniella* with a sucrose solution at 0.5 M increased fecundity of *N. tenuis* and lowered the number of *E. kuehniella* eggs consumed by the predator, which ultimately may reduce production cost. In contrast, the anthocorid *O. laevigatus* did not benefit from a supplementary 5% sucrose solution (Bonte and De Clercq, 2010a).

Noninsect materials may hold potential as factitious foods for rearing insect predators. Brine shrimps of the genus *Artemia* (Branchiopoda) are routinely used as feed in aquaculture (Lavens and Sorgeloos, 2000). Arijs and De Clercq (2001) were the first to test cysts (diapausing eggs) of the brine shrimp (*Artemia franciscana* Kellogg) as a food for a heteropteran predator. They compared the development and reproduction of *O. laevigatus* on *A. franciscana* cysts versus *E. kuehniella* eggs. Feeding this predator during a single generation on hydrated, decapsulated cysts (which had been stored in dry form) resulted in development, fecundity, and oviposition rates comparable to those of predators fed frozen *Ephestia* eggs. Later studies indicated that brine shrimp cysts were suitable food for several *Orius* spp. (Riudavets et al., 2006; Bonte and De Clercq, 2008; Nishimori et al., 2016) and for the mirids *Dicyphus errans* (Wolff) (Arvaniti et al., 2018), *N. tenuis* (Owashi et al., 2020) and *Macrolophus* spp. (Fig. 3.2) (Callebaut et al., 2004; Castañé et al., 2006; Riudavets et al., 2006; Vandekerkhove et al., 2009), but less so for the larger pentatomid predators, *P. maculiventris* and *P. bidens* (Mahdian et al., 2006). Results obtained with different predatory bugs have been known to vary as a function of decapsulation, hydration and origin of the cysts (De Clercq et al., 2005; Vandekerkhove et al., 2009). In addition, some batch-to-batch variability in performance has been observed. Whereas the predators usually can handle the cysts better when they are hydrated to some degree, the use of fully hydrated cysts leads to problems with mold in the rearing containers (Vandekerkhove et al., 2009). Moreover, prolonged rearing on cysts as a sole food has been associated with fitness losses in *Orius* bugs (De Clercq et al., 2005). Although the practical value of brine shrimp cysts as feed does not equal that of *E. kuehniella* eggs, they did find practical application as a supplementary food in the mass rearing of different predatory heteropterans. An important consideration here is that *Artemia* cysts are at least an order of magnitude cheaper than *E. kuehniella* eggs (Arijs and De Clercq, 2001). Currently, dry *Artemia* cysts are routinely mixed with *E. kuehniella* eggs to reduce inputs of expensive lepidopteran eggs in the production

FIGURE 3.2 Nymph of *Macrolophus pygmaeus* with decapsulated *Artemia franciscana* cysts. *Photo: L. Parmentier.*

process of different predatory heteropterans. Interestingly, Lu et al. (2011) found that feeding the *Artemia* cysts to *Orius strigicollis* (Poppius) nymphs and feeding *E. kuehniella* eggs to adults was an acceptable method of mass rearing this predator.

Another potential factitious food for predatory heteropterans, that are produced by the billions in mass-rearing facilities for sterile insect techniques, are tephritid fruit flies. Takara and Nishida (1981) reared *O. insidiosus* on a diet of eggs of the oriental fruit fly, *Dacus* (*Bactrocera*) *dorsalis* (Hendel), with similar developmental and reproductive performance as compared to natural food. Eggs of the medfly, *Ceratitis capitata* (Wiedemann), have been used to produce the mirid predators *Cyrtorhinus lividipennis* Reuter (Liquido and Nishida, 1985), *M. pygmaeus* (Nannini and Souriau, 2009) and *Orius* spp. (Bonte et al., 2017). Hough-Goldstein and McPherson (1996) reported that colonies of the two-spotted stinkbug *P. bioculatus* could be maintained on a combination of heat-killed larvae of the Mexican fruit fly, *Anastrepha ludens* (Loew), and the European corn borer, *Ostrinia nubilalis* (Hübner).

The easily reared and highly reproductive drosophilid fruit flies have been tested as a factitious food for *Orius*. Montoro et al., 2020b, investigated life history traits and biochemical composition of *O. majusculus* when reared in the nymphal and/or adult stage on frozen adults of *Drosophila melanogaster* (Meigen). Fruit flies produced on a protein-rich artificial diet proved to be a suitable food for nymphs of the predator, but not for adults given that their fecundity was considerably reduced as compared with a control diet of *E. kuehniella* eggs. Fruit flies reared on a lipid-rich diet were deemed inadequate for the production of *O. majusculus*.

Astigmatid storage mites are routinely used for the production of a range of predatory mites (see Chapter 7), but they have rarely been tested as a factitious prey for the smaller predatory bugs. Bonte et al. (2017) compared developmental and reproductive traits of two *Orius* spp. on various factitious prey, including the astigmatids *Tyrophagus putrescentiae* (Schrank) and *Carpoglyphus lactis* (L.). Whereas *Orius thripoborus* (Hesse) succeeded in developing and reproducing on each of these astigmatid species, albeit at a lower pace than on eggs of flour moths or medflies, *Orius naivashae* (Poppius) suffered high mortality in the nymphal and adult stage and produced no eggs when exclusively offered mite prey. *Tyrophagus putrescentiae* was also deemed a suitable prey for rearing the anthocorids *O. laevigatus* (Gomaa and Agamy, 2002), *O. sauteri* (Yang et al., 2009) and *O. insidiosus* (Bernardo et al., 2017), but not for *Orius tantillus* (Motschulsky) (Nagai et al., 1998). These studies indicate that closely related species may differ extensively in their capacity to use certain alternative prey.

Predatory bugs of the families Pentatomidae and Reduviidae have been reared using larvae and pupae of several insect orders as factitious prey. These include the larvae of the lepidopterans *Galleria mellonella* L. (Pyralidae), *C. cephalonica* (Pyralidae), *Diatraea saccharalis* (F.) (Pyralidae) and *Bombyx mori* L. (Bombycidae); larvae and pupae of the coleopterans *Tenebrio molitor* L. and *Zophobas confusa* Gebien (Tenebrionidae); and larvae of the dipterans *Musca domestica* L. (Muscidae), *Calliphora erythrocephala* Meigen and *Chrysomya putoria* (Wiedemann) (Calliphoridae) (Li et al., 1997; De Clercq, 2000 and references therein; Grundy et al., 2000; Sahayaraj, 2002; Zanuncio et al., 2001, 2005; Torres et al., 2006; Sorkhabi-Abdolmaleki et al., 2013; De Bortoli et al., 2016; Lenin and Rajan, 2016; Botteon et al., 2017; Sahid et al., 2018). De Clercq and Degheele (1993c) attributed better success for the laboratory rearing of *Podisus* bugs with larvae of the greater wax moth *G. mellonella* as compared with those of the beet armyworm *S. exigua* to the higher protein and fat content and lower water content of the former prey type. De Clercq et al. (1998b)

found that nymphal development of *P. maculiventris* was faster on pupae of the yellow mealworm *T. molitor* than on larvae of *T. molitor* or on larvae of *G. mellonella*. Male or female body weight was not affected by prey treatment, but total egg production was greatest for females reared on *G. mellonella* larvae. The authors pointed out; however, that the expense for producing yellow mealworms is lower than for wax moth larvae. Moreover, live wax moth larvae also proved more difficult to handle by the predators (mainly due to their web-spinning behavior), such that they need to be inactivated or killed before being supplied as food in the predator cultures. Guimaraes de Menezes et al. (2014) found that the diet of *T. molitor* larvae fed to *P. nigrispinus* had an effect on the population growth parameters of the pentatomid; mealworms maintained on corn meal yielded the lowest net reproductive rate of *P. nigrispinus*, whereas those given wheat brans proved to be the best diet for the predator. Life table studies by Morales-Ramos et al. (2016) suggested that yellow mealworm pupae, rather than larvae, were more suitable factitious prey for mass production of *P. maculiventris*. The superior performance of the predator when reared on pupae was likely the result of improved nutrient value, as the ratios of lipid, protein, and sugars were different between larvae and pupae. Higher protein content, lower overall lipid content, and lower polyunsaturated fatty acids in the pupal stage compared with the larval stage were believed to be more beneficial for the development and fecundity of *P. maculiventris*.

In Brazil, larvae and pupae of mealworms and larvae of houseflies have been used successfully for the rearing of different pentatomid predators, including *P. nigrispinus, Supputius cinctipes* (Stål) and *Brontocoris tabidus* (Signoret) (Zanuncio et al., 1996, 2001; Jusselino-Filho et al., 2003; Zanuncio et al., 2005; Guimaraes de Menezes et al., 2014; Campos et al., 2018; Peluzio et al., 2018). De Bortoli et al. (2011) stated that the larval stage of the housefly *M. domestica* was the most cost-effective factitious prey for mass rearing of *P. nigrispinus*. Torres et al. (2006) pointed out that the cost of producing yellow mealworms and houseflies is low, given the relatively low cost of the diet components, labor for handling the insects, rearing containers and space. Zanuncio et al. (2001) studied the development and reproduction of the Neotropical pentatomid predator *P. nigrispinus*, fed with combinations of yellow mealworm pupae and housefly larvae, either presented together, or presented on alternate days. They noted that heavier females were produced from a diet of *T. molitor* pupae alone, or from a diet of both prey on alternate days, rather than both prey simultaneously, or *M. domestica* alone. The authors further found that female body weight did not correlate with fecundity. Nonetheless, fecundity of *P. nigrispinus* was superior when the predator was maintained on mixed prey (given simultaneously or on alternate days) than when reared on *T. molitor* alone. The authors concluded that a mixed diet of *T. molitor* pupae and *M. domestica* larvae were the most suitable for the production of *P. nigrispinus*. Interestingly, Grundy et al. (2000) came to similar conclusions when rearing the assassin bug *P. plagipennis* on coddled larvae of *T. molitor* and *H. armigera*. When reared on *T. molitor* alone, *P. plagipennis* had the highest body weight, but its fecundity was substantially inferior to that on a mix of both prey. These findings again indicate the benefits of providing predatory heteropterans with mixed diets.

3.2.3 Artificial diets

3.2.3.1 Definitions

The availability of an artificial diet that optimizes the growth and reproduction of a natural enemy could advance the automation of its mass production (De Clercq, 2008; see also Chapter 8). The literature prior to 1998 on artificial diets for predatory bugs was covered by the reviews of Thompson (1999) and Thompson and Hagen (1999), whereas that published between 1998 and 2007 was reviewed by Riddick (2009). Artificial diets for insects have traditionally been classified as holidic (chemically defined), meridic (most components are known chemically), or oligidic (mainly composed of crude organic materials) (Dougherty, 1959). As the distinction between these three categories is not always clear, Grenier and De Clercq (2003) proposed a classification system separating artificial diets for insect natural enemies (predators and parasitoids) based on whether they contain insect components (i.e., tissues, hemolymph, cells, protein, amino acids, etc.) or not. Artificial diets containing insect components may be useful when predators require certain growth factors, feeding stimulants, and other chemical cues found in arthropod prey (De Clercq, 2008; Riddick, 2009).

3.2.3.2 Effect on development and reproduction

Table 3.2 provides an overview of exemplary studies that compared the life parameters of heteropteran predators fed on artificial diets versus factitious or natural prey. Cohen (1985) proposed a meat-based diet for the rearing of the big-eyed bug, *G. punctipes*, serving as a basis for several subsequent studies using meat diets for a variety of predatory bugs. The diet was a paste composed of fatty ground beef and beef liver supplemented with a 5% sucrose solution and

TABLE 3.2 Exemplary studies on the influence of consuming artificial diets vs factitious or natural prey on life history parameters of predatory heteropterans.

Predator	Artificial diet—animal protein base	Results/outcome	References
Orius laevigatus nymphs, adults (Anthocoridae)	Beef liver, ground beef—in Parafilm packets	Development time (↑), survival (↓), size (√), oviposition (√)	Arijs and De Clercq (2004)
O. laevigatus nymphs, adults	Hen's egg yolk—in Parafilm domes	Development time (↑), survival (↓), size (↓), oviposition (√), oogenesis (↓), longevity (√)	Bonte and De Clercq (2008)
Orius sauteri nymphs, adults	Hen's egg yolk, silk moth pupal homogenate—in sodium alginate–chitosan microcapsules	Development time (↑), survival (↓), fertility (↓)	Tan et al. (2013)
Orius majusculus nymphs, adults	Hen's egg yolk—in Parafilm domes	Development time (↑), survival (√), size (↓), oviposition (↓)	Montoro et al. (2020a)
Geocoris punctipes adults (Lygaeidae)	Beef liver, ground beef—in Parafilm	Size (√), oviposition (↓)	Cohen (1985)
Dicyphus tamaninii nymphs, adults (Miridae)	beef liver, fatty ground beef, hen's egg yolk—in Parafilm	Development time (↑), survival (√) by fourth–fifth generation, size (↓), oviposition (√)	Iriarte and Castañé (2001)
D. tamaninii nymphs, adults	Beef liver, fatty ground beef, hen's egg yolk, casein-reformulated diet	Development time (↑), survival (↑), size (√), oviposition (√)	Zapata et al. (2005)
Hyaliodes vitripennis nymphs, adults (Miridae)	Pork liver, whey powder—in Parafilm	Survival (↑), longevity (↑), oviposition (↑)	Firlej et al. (2006)
H. vitripennis nymphs, adults	Beef liver, ground beef, whole hen's egg—in Parafilm	Survival (↑), longevity (↑)	Firlej et al. (2006)
Macrolophus caliginosus nymphs, adults (Miridae)	Beef liver, fatty ground beef, hen's egg yolk, casein—in Parafilm	Development time (↑), survival (↓), size (↓)	Castañé and Zapata (2005)
Macrolophus pygmaeus (caliginosus) nymphs, adults (Miridae)	Hen's egg yolk—in Parafilm domes	Development time (↑), survival (√), size (↓), oviposition (↓), oogenesis (↓)	Vandekerkhove et al. (2006, 2011)
Perillus bioculatus adults (Pentatomidae)	Pork liver, fatty ground beef—in Parafilm domes	Oogenesis (↓)	Adams (2000a)
P. bioculatus adults	Pork liver—in Parafilm domes	Oviposition (↓), longevity (√)	Adams (2000b)
P. bioculatus nymphs, adults	Beef, whole hen's egg—in Mylar-Parafilm	Development time (↑), survival (↓), total oviposition (↓), longevity (↓)	Coudron and Kim (2004)
P. bioculatus nymphs, adults	Chicken liver, tuna fish—in Parafilm capsules	Development time (↑), survival (↓), oviposition (↓)	Rojas et al. (2000)
Picromerus bidens nymphs, adults (Pentatomidae)	Beef liver, fatty ground beef, hen's egg yolk—in Parafilm	Development time (↑), survival (↓), size (↓)	Mahdian et al. (2006)
Podisus maculiventris nymphs, adults (Pentatomidae)	Beef liver, fatty ground beef, hen's egg yolk—in Parafilm	Development time (↑), survival (√), size (↓), oviposition (↓), longevity (√)	De Clercq et al. (1998a), Mahdian et al. (2006)
P. maculiventris nymphs, adults	beef liver, whole hen's egg—in Mylar-Parafilm	Development time (↑), size (↓), oviposition (↓)	Wittmeyer and Coudron (2001)
P. maculiventris nymphs, adults	Beef liver, whole hen's egg—in Mylar-Parafilm	Body size (↓), oviposition (↓), oogenesis (↓), vitellogenesis (↓)	Wittmeyer et al. (2001)

(*Continued*)

TABLE 3.2 (Continued)

Predator	Artificial diet—animal protein base	Results/outcome	References
P. maculiventris nymphs, adults	Beef liver, whole hen's egg—in Mylar-Parafilm	Development time (↑), size (↓), survival (√), oviposition (↓)	Coudron et al. (2002)
Podisus nigrispinus nymphs, adults (Pentatomidae)	Beef liver, ground beef	Development time (↑), size (↓), ovarian weight (↓)	Lemos et al. (2003)
Arma chinensis nymphs, adults (Pentatomidae)	Pig liver, tuna fish—in composite Parafilm-plastic film domes	Development time (↑), survival by first–third generation (↓), survival by fourth–sixth generation (√), size (↓), oviposition (↓)	Zou et al. (2013b)

Symbols indicate decrease (↓), increase (↑), or no significant effect (√) on a given life parameter, in comparison to a control of natural or factitious prey, for designated predator.
Source: Modified from Riddick, E.W., 2009. Benefits and limitations of factitious prey and artificial diets on life parameters of predatory beetles, bugs, and lacewings: a mini-review. BioControl 54, 325–339.

wrapped in stretched Parafilm. The gross nutritional composition of this diet reflected that of the predator's natural prey, *H. virescens* eggs. The diet was supplemented with free water only and no plant materials were offered. Weight of adults and eggs of diet-fed *G. punctipes* were comparable to those of their counterparts fed insect eggs, but fecundity was lower, which was mainly attributed to egg cannibalism. The diet was cheap (c. 3 USD per kg) and allowed continuous production of the predator for over 150 generations over a time span of nearly 15 years (Cohen et al., 1999; Cohen, 2000a).

De Clercq and Degheele (1992,1993b) reported that consecutive generations of the pentatomid predators *P. maculiventris* and *P. nigrispinus* (misidentified as *P. sagitta*) could be reared on a meat-based diet modified from Cohen's (1985) diet. The main modification consisted of the addition of fresh chicken's egg yolk. In comparison to rearing on wax moth larvae, the development of the predators was prolonged by 15%–40% and adult weights were 18%–28% lower. Fecundity on the artificial diet was only about 30%–50% of that on *G. mellonella* larvae, but egg weights and egg hatch were similar. In a follow-up study on *P. maculiventris*, De Clercq et al. (1998a) confirmed that adults reared on artificial larvae composed of a meat and egg yolk diet were smaller than those produced on larvae of two factitious prey, *G. mellonella* and *T. molitor*. However, whereas fecundity of predators on the artificial diet remained inferior to that of wax moth larvae, it was similar to that on yellow mealworms. Mahdian et al. (2006) used a diet that was slightly modified from that proposed by De Clercq et al. (1998a) (ascorbic acid was omitted) for *P. maculiventris*. Their results indicated that nymphal survival and longevity on the diet were acceptable, but female body weight and oviposition rate were reduced when using the artificial diet rather than live prey such as *S. littoralis* or *G. mellonella* larvae. The meat diet developed by De Clercq & Degheele (1992), enriched with silkworm pupa powder, was also reported to be suitable for rearing another asopine predator, *Andrallus spinidens* L. (Shimizu et al., 2014).

Greany and Carpenter (1998) reported that artificial diets containing ground liver and fresh egg yolk and encapsulated in Parafilm using an encapsulation apparatus allowed egg to adult development of several predatory heteropterans, including *P. maculiventris, P. bioculatus, G. punctipes* and *X. flavipes*. Wittmeyer and Coudron (2001) reported that an artificial diet for *P. maculiventris* based on beef liver and whole egg and encapsulated in a Mylar-Parafilm was inferior to coddled cabbage looper (*T. ni*) larvae. The actual cost required to double the size of the *P. maculiventris* population, in culture, was two times greater when predators were fed exclusively on the artificial diet in the nymphal and adult stages, as compared to using *T. ni* larvae. Supplying *T. ni* larvae to adults and artificial diet to nymphs tended to improve oviposition, reproductive rate and intrinsic rate of increase, but not enough to offset the loss in egg production. Wittmeyer et al. (2001) provided further evidence that the same artificial diet was ineffective, in comparison to *T. ni* larvae, as a sole food source for maximizing egg production in *P. maculiventris* females. Their study highlighted the impact of nymphal and adult food sources on oogenesis (egg maturation) and vitellogenesis (yolk deposition) in this pentatomid predator.

Coudron et al. (2002) evaluated the effect of a blended, buffered mixture of a plant-based diet for *Lygus* bugs with beef liver and whole egg, again encapsulated in Mylar-Parafilm domes, on *P. maculiventris*. Nymphal survival was similar, but other life parameters (see Table 3.2) were negatively impacted when predators were fed the zoophytogenous

artificial diet for 1 or 11 generations compared to cohorts fed natural prey, *T. ni* larvae, for 1 generation. However, a gradual improvement in life parameters occurred from the first to the 11th generation on the artificial diet, possibly as a consequence of adaptation or selection. Based on a comparison of the cost to double the population size, the authors estimated that after 11 generations the cost of rearing *P. maculiventris* on the artificial diet was 1.2 times higher than on natural prey. Coudron et al. (2012) measured the levels of 10 trace elements in *P. maculiventris* reared on the same artificial diet versus those maintained on *T. ni* larvae. The study indicated that trace element levels in *P. maculiventris* were substantially affected by the food source and can vary significantly from levels in the food source. The authors concluded that a more thorough knowledge of mineral levels in predatory insects and their food may lead to higher-quality artificial diets.

The value of artificial diets for production of another pentatomid predator, the two-spotted stinkbug, *P. bioculatus*, was investigated by Adams (2000a,b), Rojas et al. (2000) and Coudron and Kim (2004). Adams (2000a) reported that females fed an artificial diet composed of pork liver and fatty ground beef rather than a control diet of freeze-killed *H. virescens* larvae had fewer developing follicles. Interestingly, 10-day-old females fed the artificial diet contained chorionated (mature) follicles in just 40% of their ovarioles whereas 9-day-old females fed the control diet contained chorionated follicles in 100% of their ovarioles. An average of 42 and 138 eggs were oviposited by mated females fed the artificial diet and frozen *H. virescens* larvae, respectively (Adams, 2000a). Apparently, the artificial diets did not provide an adequate supply of nutrients essential for egg development in *P. bioculatus*.

In another study on *P. bioculatus*, Rojas et al. (2000) formulated two meridic diets to reflect the nutritional composition of eggs of the Colorado potato beetle, *L. decemlineata*. They modified the first diet (diet 1) with a substitution of chemically defined components to produce the second diet (diet 2). The main animal protein sources of these diets were tuna fish, chicken liver, hen's egg and baby milk; they also contained soybean hydrolysate and yeast hydrolysate. In comparison to control of *L. decemlineata* eggs, both artificial diets were not satisfactory for timely development and survival of *P. bioculatus* nymphs or oviposition and longevity of adults. After 11 generations, females fed diet 2 weighed more than cohorts fed the natural prey. The authors observed that larger predators resulted after feeding on an artificial diet for many generations, suggesting that some adaptation had occurred resulting in greater ingestion of the diet.

Coudron and Kim (2004) could culture *P. bioculatus* on an artificial diet based on animal protein (beef, hen's egg) and undisclosed plant protein for 11 generations, but the diet was substandard to natural prey (*T. ni* larvae). Nevertheless, the cost associated with culturing *P. bioculatus* on artificial diet or natural prey was about the same. A meat-based artificial diet was inferior for rearing another pentatomid *P. bidens* as compared to natural and factitious prey (Mahdian et al., 2006). Adult females were heavier on lepidopteran larvae (the cotton leafworm, *S. littoralis* and the greater wax moth *G. mellonella*) than on the artificial diet. The total fecundity of *P. bidens* on *S. littoralis* larvae was superior to that on *G. mellonella*; *P. bidens* did not produce eggs when fed the artificial diet.

The Neotropical pentatomid *P. nigrispinus* benefited less when fed an artificial diet composed of beef liver and ground beef rather than when given natural or factitious prey (Lemos et al., 2003). Nymphs of the predator reared on an artificial diet weighed significantly less than their counterparts fed cotton leafworm, *A. argillacea*, larvae. Further, fresh weight of ovaries from newly emerged *P. nigrispinus* females was lower for individuals reared on an artificial diet than for those produced on yellow mealworm (*T. molitor*), housefly (*M. domestica*), or cotton leafworm larvae. Lemos et al. (2005) confirmed that food source has a profound impact on ovarian development. The latter study showed that newly emerged *P. nigrispinus* females had ovaries containing oocytes in an advanced, intermediate, or early stage of development when fed *A. argillacea* larvae, *T. molitor* or *M. domestica* larvae, or a meat-based artificial diet, respectively.

Zou et al. (2013b) developed an insect-free artificial diet comprised of pig liver and tuna for *Arma chinensis* (Fallou), a pentatomid predator that is native to China, Korea and Mongolia. Several life history parameters were diminished when the predator was reared on the artificial diet as compared with pupae of the Chinese oak silk moth, *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae). Fecundity and egg viability were lower, developmental time from second instar to adult and the preovipositional period were significantly longer for diet-fed *A. chinensis*. Nymphal weight, body length, adult longevity, survival from second instar to adult, and fertility increased, while sex ratio (male:female) decreased, with the rearing of consecutive generations on the diet. Additionally, the longevity of adults reared on the artificial diet was significantly longer than of those reared on pupae. As a result, the cost to rear *A. chinensis* on the artificial diet approached 2.0 times the cost of rearing the pentatomid on pupae of *A. pernyi* (Zou et al., 2015). The use of nutrigenomics to assess performance (Zou et al., 2013a) and to assist in diet formulation (Zou et al., 2019) for *A. chinensis* are discussed in Chapter 8.

Given their economic importance as biocontrol agents, there has been extensive work on artificial diets for *Orius* spp. Zhou and Wang (1989) designed a diet, to sustain the development of *O. sauteri*, composed of brewer's yeast, egg yolk, a soy protein acid hydrolysate, sucrose, honey, palmitic acid and water. The diet yielded good nymphal survival, but

development was prolonged as compared to natural and factitious prey. Ferkovich and Shapiro (2004a) reared *O. insidiosus* on a diet based on that of Zhou and Wang (1989) supplemented with cells from an embryonic cell line, IPLB-PiE, of the Indian meal moth *P. interpunctella*. The diet was offered to the bugs in Parafilm domes produced with an encapsulation device (Greany and Carpenter, 1998). It was found that supplementation using the PiE cell line increased egg production. Oviposition rate increased as the concentration of cells added to the diet increased from 0.25 to 0.75 mL of PiE cells/mL of artificial diet. A follow-up study investigated the effects of insect prey-derived and noninsect derived supplements, added to the Zhou and Wang (1989) diet, on egg production and oviposition rate of *O. insidiosus* (Ferkovich and Shapiro 2004b). Proteins derived from *P. interpunctella* eggs were found to be superior for *O. insidiosus* in terms of egg production and oviposition as compared to proteins from vertebrates (bovine serum albumin, chicken liver, beef liver, chicken egg albumin). The concentration of *P. interpunctella* egg proteins necessary for satisfactory oviposition rates was 80-fold less than that of vertebrate-derived proteins needed for the same purpose.

Ferkovich and Shapiro (2005) fractionated *E. kuehniella* eggs to identify the most active fraction via feeding bioassays with *O. insidiosus*. They noted that egg production of the predator increased only when a fraction with a pH of 5 was included. According to the authors, this finding points to the presence of a specific nutritional factor in certain lepidopteran eggs that enhances fecundity of predators. Ferkovich and Lynn (2005) supplemented the artificial diet of Zhou and Wang (1989) with cells from an embryonic cell line Ek-x4V, from *E. kuehniella* eggs. Adults of *O. insidiosus* fed the supplemented diet had higher oviposition rates than those fed the unmodified artificial diet, but their oviposition rates were inferior to those of adults fed *E. kuehniella* eggs. Likewise, oviposition of *O. insidiosus* was enhanced when artificial diet was supplemented with protein from *E. kuehniella* eggs in comparison to unmodified artificial diet (Ferkovich et al., 2007). Yet, the modified artificial diet was substandard to intact *E. kuehniella* eggs.

Lee and Lee (2004) reared the anthocorid *O. strigicollis* on an artificial diet composed of powdered pupae of the Chinese oak silkworm, *A. pernyi*, beef liver, beef powder, and chicken egg yolk. When fed this diet, the development time and survival of the predator were 14 days and 68.5%, respectively. Total fecundity averaged 82.5 eggs during an oviposition period of 18 days. Unfortunately, the authors did not compare the performance of the predator on the artificial diet versus natural or factitious prey. Using an orthogonal design, Tan et al. (2013) tested different combinations of egg yolk, and whole-pupa homogenate of the Tussah silk moth (*Antheraea paphia* L.), honey, sucrose, rapeseed pollen and sinkaline (choline chloride) for *O. sauteri*. The authors investigated the developmental and reproductive performance of the predator, as well as respiratory and locomotory parameters and predation potential. Overall, rearing of *O. sauteri* on microcapsules containing the artificial diet slowed down development and decreased nymphal survival, fecundity and respiratory rate of adult females as compared with rearing on natural prey (*Tetranychus cinnabarinus* Boisduval). The pupal homogenate of *A. paphia* was considered to be a key component of the artificial diet, particularly as it boosted the nymphal development of *O. sauteri*.

Arijs and De Clercq (2004) used a deletion and addition approach to modify the meat-based diet developed by De Clercq et al. (1998a) for *P. maculiventris* and used it for rearing *O. laevigatus* (Fig. 3.3). They found that beef liver was the most important component to support development and reproduction of the anthocorid, whereas egg yolk, ground beef, ascorbic

FIGURE 3.3 Adult of *Orius laevigatus* feeding on a meat-based artificial diet wrapped in Parafilm. *Photo: Y. Arijs.*

acid and sucrose were of minor importance. Predators supplied with the different artificial diets tested in this study developed slower than those given *E. kuehniella* eggs but their adult weights were similar. Fecundity of females provided with diets containing beef liver was similar to or somewhat lower than that of females fed lepidopteran eggs. Oviposition rate and egg hatch were not affected by diet. Bonte and De Clercq (2008) compared different artificial diets to two factitious foods, *E. kuehniella* eggs and *A. franciscana* cysts, for the rearing of *O. laevigatus*. Overall, the factitious foods proved superior to the artificial diets, and a meat and liver diet yielded better results than three diets based on egg yolk. Interestingly, within the egg yolk diets, the developmental fitness of the anthocorid varied proportionally with the amount of egg yolk present in the diet. Bonte and De Clercq (2011) reported that access to a bean pod had a positive influence on the development of *O. laevigatus* nymphs reared on an egg yolk-based artificial diet. Montoro et al. (2020a) compared development and reproduction of *O. majusculus* on artificial diets with that on *E. kuehniella* eggs. They used an egg yolk-based diet tested earlier for *M. pygmaeus* (Vandekerkhove et al., 2006) and *O. laevigatus* (Bonte and De Clercq, 2008) and made variations in its lipid/protein content yielding six different artificial diets. Whereas nymphal survival was not affected by diet, development was slightly delayed on the artificial diets. However, female body mass and fecundity were significantly lower on all the artificial diets compared with the diet of lepidopteran eggs. Among the artificial diets, females fed a lipid-rich diet laid more eggs than those offered high protein diets. Overall, variations in protein and/or lipid content in the carcass of the predator were unrelated to differences in fitness parameters.

Rajabpour et al. (2018) tested an artificial diet based on (lamb) liver and egg yolk and containing an extract of *E. kuehniella* eggs for the related anthocorid *O. albidipennis*. They found that survival and fecundity of the predator were lower than on a control diet of *E. kuehniella* eggs and pollen.

An artificial "pudding" diet was designed by Li et al. (2013) for the anthocorid *Tetraphleps galchanoides* Ghauri, a natural enemy of the hemlock woolly adelgid, *Adelges tsugae* (Annand). The diet contained low-fat milk powder, pine pollen, honey, sugar, brewer's yeast, potassium sorbate and distilled water. Although survival of the *T. galchanoides* nymphs was lower on the artificial diet than on *A. tsugae*, developmental rates and fecundity were similar and egg hatch and adult longevity were even superior to that on the natural prey.

Artificial diets have been tested for mirid predators, with variable results. Grenier et al. (1989) modified an artificial diet developed for *Trichogramma* egg parasitoids for the rearing of *M. caliginosus* nymphs. They did so by supplementing a basic diet containing organic acids, amino acids, sugars, minerals, vitamins, albumin and egg yolk with different vertebrate sources of proteins and lipids. The artificial diets were offered in floral foam enveloped in Parafilm. Best results were obtained when the basic diet was supplemented with chicken embryo extract, yielding a nymphal survival of 21% (vs 33% in the control on *E. kuehniella* eggs). However, when the latter artificial diet was supplemented with leaves of *Pelargonium peltatum* L., 62% of the nymphs reached the adult stage. Similarly, the development and fecundity of the related species *M. pygmaeus* were better on an egg yolk-based diet supplemented with honeybee pollen than on the artificial diet alone indicating the importance of plant-feeding for these mirids.

Castañé and Zapata (2005) tested the value of a meat-based artificial diet for culturing *M. caliginosus*. Predators reared on the meat diet had smaller hind tibiae, weighed less, and took longer to develop than their counterparts reared on *Ephestia* eggs. Nevertheless, the artificial diet allowed the production of continuous generations of the mirid in the absence of plant material (as a moisture source or oviposition substrate). Vandekerkhove et al. (2006) investigated whether artificial diets based on hen's egg yolk could provide adequate nutrition for the development and reproduction of *M. pygmaeus* (named as *M. caliginosus*). They reported that the artificial diets, encapsulated in Parafilm domes (Fig. 3.4), resulted in prolonged development and lower adult weights as compared with *E. kuehniella* eggs, but survival was similar. Further, dissection of females on day 7 indicated that females fed *Ephestia* eggs had more developing eggs (oocytes) in their ovaries than those fed an artificial diet. Callebaut et al. (2004) also found that *Ephestia* eggs were more suitable than artificial diets for *M. caliginosus* reproduction via an examination of egg load. In the studies by Callebaut et al. (2004) and Vandekerkhove et al. (2006) on *Macrolophus* bugs and Bonte and De Clercq (2008) on *O. laevigatus*, egg load correlated significantly with oviposition rate. This result indicated that counting oocytes in females in the early adult stage constitutes a reliable and fast tool for assessing the reproductive potential of these and perhaps other predatory bugs.

Iriarte and Castañé (2001), Castañé et al. (2002), and Zapata et al. (2005) conducted diet-related research on *Dicyphus tamaninii* Wagner, another mirid predator of whiteflies and other small arthropods found in the Mediterranean area. Iriarte and Castañé (2001) found that *D. tamaninii* is amenable to rearing, during its nymphal and adult stages, on a meat-based artificial diet devoid of plant material. Despite an initial reduction of survival of individuals in the first generation, an incremental increase in survival occurred with succeeding generations reared on the artificial diet. In an attempt to increase the suitability of a meat-based artificial diet for the mirid, Zapata et al. (2005) conducted a biochemical analysis of the carcass of diet-reared adults. They detected nutritional deficiencies in the original diet formulation by comparing the total, free amino acid and lipid profiles of females reared on the meat diet versus

FIGURE 3.4 Nymph of *Macrolophus pygmaeus* feeding on a Parafilm dome containing an egg yolk diet. *Photo: T. Machtelinckx.*

control females fed *E. kuehniella* eggs. To remediate the nutritional deficiencies, Zapata et al. (2005) added new sources of protein (aspartate, casein) and fatty acids (soybean oil) to the original diet, and more water to lower the concentration of all components and improve diet acceptability. This reformulated artificial diet was more beneficial to the predators as evidenced by an increase in the survival of nymphs and the body weight of adults. Adults fed the reformulated diet had hind tibia of similar lengths as cohorts reared on *Ephestia* eggs. Oviposition rates did not differ between control and artificial diets.

Firlej et al. (2006) observed promising effects of artificial diets on another mirid *Hyaliodes vitripennis* (Say), which is an indigenous generalist predator of several arthropod pests including mites in apple orchards in North America. Two meat-based diets (originally designed for the coccinellid *Coleomegilla maculata* (DeGeer) and the chrysopid *Chrysoperla rufilabris* (Burmeister)) increased nymphal survival and adult longevity of the predator in comparison to individuals reared on (one of its) natural prey, the two-spotted spider mite *Tetranychus urticae* Koch. Adults reared on the coccinellid diet had higher oviposition rates than those reared on the chrysopid diet or on *T. urticae*. It is not clear whether the better performance of the mirid on the artificial diet as compared with *T. urticae*, means that the diet is optimal for reproduction or that *T. urticae* is a poor natural prey. The addition of a possible phagostimulant, the plant sterol β-sitosterol, to the coccinellid diet decreased nymphal mortality and slightly increased weight gain in comparison to cohorts reared on the coccinellid diet alone. The addition of sucrose to the coccinellid diet did not have beneficial effects. The authors concluded that further diet improvements are necessary to increase egg hatch and fecundity of females reared on either one of the meat diets from the first instar.

Finally, reduviid predators have been cultured on artificial diets. Sahayaraj et al. (2006) reared *Rhynocoris* spp. on a basic artificial diet containing beef extract, milk powder and egg yolk supplemented with different proportions of insect materials, pig liver or pig blood. Behavioral experiments showed that diets containing pig liver were preferred by the predators. Tomson et al. (2017) described a rearing method for *Rhynocoris fuscipes* F. based on a mixture of natural prey and a liquid artificial diet containing powdered beef liver, egg yolk, honey and water as the main components.

In summary, artificial diets tested for culturing predatory bugs were variable in composition, but usually consisted of vertebrate protein from beef and chicken and, infrequently, from pig, lamb or fish components. The beef liver was a component in the artificial diet of 12 of 19 species, and hen's egg (whole or yolk only) was a component in the diet of 17 of 19 species. In several studies, adding insect components or extracts to the artificial diet yielded better results, indicating that this might have improved the nutritional value and/or acceptability of the diet to the predator. In most cases, however, artificial diets were inferior as a stand-alone food source for predatory bugs as compared with factitious or natural prey. Rarely were artificial diets as suitable (e.g., Zapata et al., 2005; Li et al., 2013) or perhaps even more suitable (Firlej et al., 2006) than factitious or natural prey to support development and reproduction of predatory bugs.

3.2.3.3 Effect on predation potential

Although developmental and reproductive parameters are useful to assess the quality of a natural enemy produced on an artificial (or factitious) food, the ultimate quality parameter is its effectiveness as a biological control agent.

Demonstrating that a predator retains the capacity to capture, kill, and consume live (target) prey, despite continuous culturing on an (inanimate) artificial diet, is indeed one of the most relevant measures of predator quality (Grenier and De Clercq, 2003).

De Clercq and Degheele (1993b) cultured the pentatomids *P. maculiventris* and *P. nigrispinus* (*P. sagitta*) for over 15 consecutive generations exclusively on artificial larvae consisting of a meat-based artificial diet encapsulated in stretched Parafilm. When presented with live prey, nymphs and adults of both species had similar predation rates on caterpillars of *S. exigua* as their counterparts maintained on *G. mellonella* larvae. Similarly, Chocorosqui and De Clercq (1999) reared *P. maculiventris* for three consecutive generations on similar meat-based artificial diet. Despite the smaller size of nymphs and adults reared on artificial diet rather than factitious prey (*G. mellonella* larvae), predation rate was unaffected. Diet-reared predators killed as many or even slightly more beet armyworm (*S. exigua*) larvae than cohorts on factitious prey. Higher predation rates of artificial diet-reared predators versus those reared on factitious prey were also reported for *P. nigrispinus* (Saavedra et al., 1997).

Predation potential of the big-eyed bug, *G. punctipes*, was compared between "domesticated" (i.e., laboratory-reared on an artificial diet for 60 continuous generations) versus F_1 progeny of feral (wild) females (Cohen, 2000a). Progeny of wild females were fed *H. virescens* eggs and heat-killed *S. exigua* larvae in the laboratory. In the predation experiments, females in both treatments were supplied with *H. virescens* larvae or pea aphids (*A. pisum*). Although domesticated females were smaller than wild females, consumption rates were approximately the same between the two treatment groups, regardless of the prey species (Cohen, 2000a).

Castañé et al. (2002) proved that *D. tamaninii* cohorts reared on a meat diet were just as effective as those reared on *E. kuehniella* eggs at killing two of its main target prey, the greenhouse whitefly [*Trialeurodes vaporariorum* (Westwood)] and the cotton aphid (*Aphis gossypii* Glover). Castañé and Zapata (2005) reported that *M. caliginosus* adults of the 7th generation of rearing on a meat-based diet and on artificial moisture and oviposition sources, were as adept at killing the greenhouse whitefly (*T. vaporariorum)*, sweet potato whitefly [*Bemisia tabaci* (Gennadius)], and two-spotted spider mite (*T. urticae)* as those reared on *E. kuehniella* eggs. Vandekerkhove et al. (2011) found that fifth instars of another mirid, *M. pygmaeus*, had lower body weights when fed on egg yolk-based artificial diets, but killed similar numbers of prey (*Myzus persicae nicotianae* Blackman) as their counterparts fed *E. kuehniella* eggs. Likewise, Bonte and De Clercq (2010b) reported that despite a lower body weight, *O. laevigatus* fifth instars and adults reared on an egg yolk-based artificial diet killed as many second instars of the target prey, *Frankliniella occidentalis* Pergande, as their peers cultured on *E. kuehniella* eggs. Tan et al. (2013) observed that adult female *O. sauteri* reared on microencapsulated artificial diet killed similar numbers of thrips and spider mites as those maintained on mite prey.

Consumption of a meat-based artificial diet did not reduce the capacity of the reduviid *R. marginatus* to kill its natural prey *S. litura* in the laboratory (Sahayaraj and Balasubramanian, 2009). Although the authors did not describe the artificial diet, pig liver, beef extract, and egg yolk were the likely sources of protein in it, since one of the authors at the same research center used a meat-based diet composed of these components to rear *R. marginatus* (see Sahayaraj et al. (2006)).

These studies suggest that even after long-term rearing on inanimate artificial diets, heteropteran predators have little difficulty switching to natural or target prey and do not have impaired predation abilities as compared with counterparts maintained on nutritionally optimal factitious prey. Several of these studies also indicate that body size (weight) is not a reliable predictor of a predator's killing capacity. However, it is noteworthy that in several of the above studies the factitious food given to the control groups for comparison also consisted of immobile insect materials. Henaut et al. (2000) reported that adults of the anthocorid *O. majusculus*, which were reared in their nymphal stages on *E. kuehniella* eggs were less effective as predators of pea aphids than their aphid-reared peers. Carvalho et al. (2011) and Castañé et al. (2014) found that *O. laevigatus* maintained on *E. kuehniella* eggs did not respond to volatiles from thrips-infested plants. These studies indicate that experience with an (inanimate) alternative food, be it a factitious prey or an artificial diet, may generate behavioral changes that affect the predator's efficacy as a biological control agent.

3.2.3.4 Challenges for the practical use of artificial diets

In 1999, Cohen et al. pointed out that despite several decades of research on artificial diets for entomophagous insects and mites, no commercial mass-rearing programs were in fact using them. Despite additional research and a few patent applications for artificial diets that could also be used for the production of predatory bugs (e.g., Cohen, 1999, 2003; White et al., 2001), the situation has remained largely unchanged. This is in sharp contrast to the extensive rearing of phytophagous insects on artificial diets. Besides the lower quality of predators reared on artificial diets as outlined above, there are many practical issues that complicate the adoption of artificial diets in commercial production systems for entomophagous insects (aptly reviewed by Cohen et al., 1999; see also Chapter 8).

A major practical downside of artificial diets proposed for culturing predatory heteropterans is their high propensity for chemical and microbial spoilage. Cohen et al. (1999) noted that the extra-oral digestion of predatory bugs tends to accelerate spoilage because of microbial and enzymatic contamination. Removal of spoiled feeds from the rearing containers is time-consuming and increases production costs. The problem can be alleviated by sterilizing the diet or by incorporating antimicrobials and antioxidants (see Cohen, 2004 for detailed methods). For instance, packages of a meat and liver diet using Parafilm as wrapping material require daily replacement to prevent a decline in nymphal survival in *O. laevigatus*. When gentamycin sulfate was added to the diet at 0.05%, diet packages could be kept in the rearing containers for 3 days without detrimental effects on the development of the predator (Arijs et al., 2002). Inglis and Cohen (2004) reported that microbial spoilage of meat diets was primarily caused by lactic acid bacteria. Latter authors also found that the antibacterial agents' streptomycin sulfate and chlortetracycline and the antifungal agents' propionic acid and potassium sorbate were highly effective in reducing the growth of microbial populations associated with the different organic components of a meat-based diet. Also, fresh egg yolk-based diets encapsulated in Parafilm (e.g., Vandekerkhove et al., 2006) easily spoil and need to be replaced at least every two days for optimal results with *Macrolophus* and particularly *Orius* bugs. In order to extend its shelf life, Goussaert (2003) lyophilized an egg yolk-based diet and rehydrated it before feeding to *O. laevigatus*. Developmental performance of the anthocorid was similar on a fresh and rehydrated diet.

More work is needed on the presentation of diets for predatory heteropterans. Most, if not all, of the diets tested for predatory bugs were encased in (stretched) Parafilm. This waxy film is readily accepted by various hemipterans (carnivorous and herbivorous species alike) as a feeding membrane. The Parafilm coating provides a firm surface that facilitates the penetration and stabilization of the piercing-sucking mouthparts of predatory bugs (Cohen, 1990; De Clercq et al., 1998a). Furthermore, it appears to slow down spoilage and dehydration of the encapsulated diet. However, packaging of diets with Parafilm has proven labor intensive even when using semiautomated systems, like the encapsulation device described by Greany and Carpenter (1998). More effective forms of encapsulation could stimulate the use of artificial diets, but few successes have been reported for heteropteran predators. De Clercq et al. (1998a) developed a gelled form of a meat-based diet for *P. maculiventris* but the results were inferior to packaging the diet in Parafilm sheets. Igarashi and Nomura (2013) performed a similar experiment with *Geocoris varius* (Uhler), and the outcome was quite similar. Tan et al. (2010) microencapsulated an artificial diet for *O. sauteri* using sodium alginate and chitosan. They reported the best microencapsulation results with 1% sodium alginate, 0.8% chitosan and a proportion of core material to wall-forming material of 1:1. In later studies on the same predator, they adjusted this to 2% sodium alginate, 0.6% chitosan and a 13:1 ratio of core material to wall-forming material, resulting in microcapsules with an average diameter of 0.65 mm (Tan et al., 2013). The microcapsules were readily accepted by nymphs and adults of *O. sauteri* and could reportedly be stored at around 10°C for over 3 months while maintaining the physical integrity of the encapsulation material and the quality of the liquid diet contained within. Similar microcapsules also proved to be suitable for the lygaeid *Geocoris pallidipennis* (Costa) (Khanzada et al., 2019). Hydrocapsules (ARS, Gainesville, USA) have been proposed for encapsulating artificial media, but besides a patent (Toreki et al., 2004) there is no published record of their efficacy for rearing predatory bugs. Finally, Ferkovich et al. (2007) pointed out that besides the diet containment method, the size and shape of individual diet units also may have a direct effect on the performance of a predator in an artificial rearing system.

Several of the studies discussed above indicate that phagostimulant components derived from insect prey or plants may enhance the acceptability of artificial diets to heteropteran predators. Indeed, a lack of feeding stimulants may be at least as crucial as a lack or poor balance of nutrients in a predator's diet (De Clercq et al., 1998a; Cohen, 2004; Ferkovich et al., 2007). Interestingly, Torres et al. (1997) and Saavedra et al. (2001) showed that brushing over the Parafilm casing of artificial larvae with a crude extract of the predator's (male) dorso-abdominal glands (DAGs) or with synthetic DAG-pheromone reduced the time for location and feeding on the artificial larvae by *P. nigrispinus*. This discovery illustrates the potential of semiochemicals to improve the attractiveness and acceptability of artificial diets for heteropteran predators and enhance their practical value in production systems.

3.3 Plant materials and alternatives

3.3.1 Plant substrates

The zoophytophagous predators of the order Heteroptera display varying degrees of plant feeding. They probe plants in the first place to fulfill their need for water: in many cases, water extracted from prey is insufficient to meet the predators' metabolic needs and water may be required to enable extra-oral digestion. In addition, some predatory bugs

acquire supplementary nutrients from plants (Naranjo and Gibson, 1996; Coll, 1998; Gillespie and McGregor, 2000; Coll and Guershon, 2002; Lundgren, 2009, 2011). Further, the female adults of predatory heteropterans may use plants as oviposition substrates (Coll, 1998; Lundgren, 2011).

The influence of supplementary plant materials on the developmental and reproductive performance of predatory bugs varies among studies (see Naranjo and Gibson, 1996; Coll, 1998 and Lundgren, 2009, 2011 for reviews). The benefit of plant-feeding appears to be dependent on the quality of insect prey and plant material used and can be species- or stage-specific (Naranjo and Gibson, 1996). The suboptimal nutritional value of certain artificial diets may in part be compensated by feeding on plant material like *Pelargonium* leaves or potato sprouts with *M. caliginosus* (Grenier et al., 1989; Castañé and Zapata, 2005), pollen with *M. pygmaeus* (Vandekerkhove and De Clercq, 2010) and bean pods with *O. laevigatus* (Bonte and De Clercq, 2011).

Overall, supplementing prey-only (insect) diet with plant material accelerates nymphal development, increases nymphal survival and adult longevity and enhances fecundity (Coll, 1998; Malaquias et al., 2010; Lundgren, 2011; Zanuncio et al., 2011). However, it is less clear whether plant material is a source of nutrients versus a source of free water. For instance, no differences in developmental and reproductive parameters of *G. punctipes* were detected between individuals fed bean pods as a supplement to insect eggs and those provided with a supplementary source of free water (Cohen and Debolt, 1983). Gillespie and McGregor (2000), however, found that nymphs of the mirid *Dicyphus hesperus* Knight provided with *E. kuehniella* eggs and tomato leaves had shorter developmental times than those supplied with flour moth eggs and wet cotton. Similar findings were reported by Arvaniti et al. (2018) for the related *D. errans*. Pods of green bean (*Phaseolus vulgaris* L.) are routinely used as a moisture source and oviposition substrate, in addition to insect prey, in *Orius* spp. cultures. Note that green bean pods (as compared to free water) can have positive, neutral, or negative effects on the development and/or reproduction of *Orius* spp. (Naranjo and Gibson, 1996; Bonte and De Clercq, 2010a, 2011; Bonte et al., 2012).

Some species of anthocorids and mirids benefit from feeding on pollen (Salas-Aguilar and Ehler, 1977; Kiman and Yeargan, 1985; Richards and Schmidt, 1996a; Lundgren, 2009; Vandekerkhove and De Clercq, 2010; Bonte et al., 2012). Pollen (usually bee-collected pollen) is included in the diet of some cultured species (e.g., Ferkovich et al., 2007; Venkatesan et al., 2008; Bonte et al., 2012). However, research shows that there is considerable variation in the performance of anthocorid predators (mainly *Orius* spp.) on pollen. This could be due to differences in the nutritional quality and defensive properties of the different pollen species involved in the studies (Richards and Schmidt, 1996a; Lundgren, 2009). In addition, the use of pollen in insect cultures has some practical drawbacks as its quality tends to deteriorate quickly and particularly when offered fresh, pollen is prone to fungal contamination.

For heteropteran predators that insert their eggs into the plant tissue (anthocorids, mirids, nabids), plants or plant parts are an essential part of the production system. Several plant materials are oviposition substrates for anthocorid, mirid and nabid predators. These include pods and stems of green bean, plant seedlings (e.g., cotton, soybean, sharp pepper), sprouts (e.g., potato, kidney bean, broad bean, soybean, alfalfa) and inflorescences (e.g, farmer's friend, *Bidens pilosa* L.) for anthocorids and nabids (Richards and Schmidt, 1996b; Coll, 1998; Murai et al., 2001; Bueno et al., 2006; Ito, 2007; Bonte and De Clercq, 2010a; Tan et al., 2014; Pascua et al., 2019). Tobacco plants or leaves have been used for predatory mirids (e.g., Fauvel et al., 1987; Constant et al., 1996; Sanchez et al., 2009; Vandekerkhove et al., 2011). Several studies have directly compared the suitability of different plant materials as oviposition substrates for predatory bugs. Braman and Yeargan (1988) noted that *Nabis* bugs tended to deposit more eggs in soybean seedlings than in green beans. Zhou et al. (1991) noted a higher oviposition rate of *O. sauteri* in soybean sprouts than in bean pods or shoots of *Forsythia suspensa* (Thunb.) Vahl. Alauzet et al. (1992) reported that *O. majusculus* deposited more eggs in leaves of geranium (*P. peltatum*) than in those of ivy (*Hedera helix* L.). Constant et al. (1996) reported similar oviposition rates by *M. caliginosus* in different parts of tobacco and geranium. Richards and Schmidt (1996b) found that although bean stems, bean pods, and potato sprouts are all suitable oviposition substrates for *O. insidiosus*, bean stems were preferred by the predator in choice tests. Moreover, the bean stems were less susceptible to mould than bean pods. The mould negatively affected the survival of hatchlings. Nonetheless, pods of green bean remain the most widely used material for mass rearing of *Orius* spp.

Besides their beneficial role as moisture sources or oviposition substrates, plant materials in predator cultures may reduce cannibalism by providing hiding places (Coll, 1998; Bonte and De Clercq, 2010a; see also below). Plant parts may also be useful for storage, shipping or application of predatory bugs (Coll, 1998). For instance, buckwheat hull is used in cultures of *Orius* bugs (e.g., Thomas et al., 2012), and as a carrier (in a mixture with vermiculite) in commercial packagings of these predators. Ito (2007) furnished rearing cages for *Orius* spp. with wheat grains to prevent cannibalism and excessive moisture.

3.3.2 Artificial substrates

In order to meet the need for plant material, large surfaces of greenhouses or open fields must be available for growing plants, or plant material must be purchased on the market. Moreover, plant material must be of good quality and free of pesticides. Thus, the requirement for plant material reduces the cost-effectiveness, reliability, and simplicity of a production system (Castañé and Zalom, 1994; Bolckmans, 2007). The elimination of plants from the rearing system of heteropteran predators requires the availability of alternative moisture sources and artificial living and oviposition substrates (Vandekerkhove et al., 2011). Most heteropterans that deposit eggs on plant surfaces in nature (pentatomids, reduviids, and geocorids) readily accept artificial substrates for oviposition, like absorbent paper toweling, mesh fabric, cotton balls or cotton wadding, precluding the need for host plants in the rearing system.

However, the situation is more complex for heteropterans that naturally insert their eggs into plant tissues. Several workers have proposed artificial oviposition substrates for anthocorids, mirids and nabids. Shimizu and Hagen (1967) observed that two anthocorids (*Anthocoris antevolens* White and *Orius tristicolor* (White)), a nabid (*N. americoferus*) and a mirid (*L. hesperus*) oviposited into Parafilm-wrapped blocks of water-soaked cellulose sponge. The Parafilm had to be stretched for the anthocorids, but not for the nabid. No data were given regarding hatching rates. Parajulee and Phillips (1992) tested water-soaked cotton dental rolls as oviposition substrates for the anthocorid *Lyctocoris campestris* (F.). The predator oviposited into the rolls but hatching of the eggs was poor. Better results were obtained with stacks of water-saturated Whatman filter papers. Castañé and Zalom (1994) developed an artificial oviposition substrate for *O. insidiosus* by covering carrageenan (Gelcarin) with paraffin wax or Parafilm. The Parafilm coating proved inadequate, but positive results were obtained when Gelcarin was covered with paraffin wax. Females successfully oviposited in the latter substrate and rates of oviposition, egg hatch, and nymphal development were similar to those on green beans. However, the authors noted that the thickness of the paraffin layer was critical, and no eggs were found when the layer was thicker than 0.045 mm. Richards and Schmidt (1996b) reported similar problems when their agar-based oviposition packets for *O. insidiosus* were covered with either stretched or unstretched Parafilm. When the Parafilm was not stretched, the bugs were unable to penetrate the surface with their ovipositor, leaving any eggs laid on the surface to dessicate. On the other hand, when the Parafilm was stretched, the eggs did not attach to the Parafilm layer and sank into the medium, causing the nymphs to drown upon hatching. Shapiro and Ferkovich (2006) reported that water-filled domes from Parafilm prepared with an encapsulation apparatus sufficed to harvest small quantities of viable eggs of *O. insidiosus;* however, the eggs had to be extracted from the domes to allow hatching. De Puysseleyr et al. (2014) investigated the effect of a plantless rearing system on the developmental and reproductive fitness of *O. laevigatus*, using wax paper as a walking substrate, water encapsulated in Parafilm domes, and an artificial oviposition substrate made of Parafilm and moist cotton wool. Plantless rearing during four generations resulted in somewhat lower adult body weights and a prolonged preoviposition period, but other biological parameters were not negatively affected by the absence of plants. In addition, plantless-reared females had similar predation rates on *F. occidentalis* larvae as their peers maintained on plants.

Constant et al. (1996), Iriarte and Castañé (2001), and Castañé and Zapata (2005) used moistened dental cotton rolls wrapped in stretched Parafilm as oviposition substrates for mirid predators. Constant et al. (1996) reported that egg laying by *M. caliginosus* was substantially increased when spraying the substrates with an ethanol extract of *Inula viscosa* (L.) Ait., a preferred host plant of this mirid. Iriarte and Castañé (2001) produced more than 5 continuous generations of *D. tamaninii* using artificial larvae containing a meat diet as food and Parafilm-wrapped dental cotton rolls dipped in green bean extract as oviposition substrates. Castañé and Zapata (2005) succeeded in rearing seven generations of *M. caliginosus* using the same artificial oviposition substrate and diet, without any detrimental effects on the bug's predation abilities. Vandekerkhove et al. (2011) cultured *M. pygmaeus* on *E. kuehniella* eggs but without plants, using an oviposition substrate modified from that of Constant et al. (1996) for over 30 consecutive generations. Parameters of development, reproduction and predation capacity of predators from the plantless rearing system were similar to those of predators from a control group maintained on tobacco leaves. Using similar methods, De Puysseleyr et al. (2012) succeeded in producing continuous generations of another mirid, *N. tenuis*, without plants (Fig. 3.5). Plantless rearing for 5 generations using *E. kuehniella* eggs as a food led to a prolonged preoviposition period and lower egg hatching rates and adult weights, but other developmental and reproductive parameters were not adversely affected.

Water is often supplied via moistened cotton wool or paper toweling. Parafilm domes produced with an encapsulation apparatus (Greany and Carpenter, 1998) have also been used as a source of water for *Orius* and *Macrolophus* bugs (e.g., Ferkovich and Shapiro, 2004a,b; Bonte and De Clercq, 2010a; Vandekerkhove et al., 2011; Bonte et al., 2012; De Puysseleyr et al., 2014). An elegant way of supplying water is via Hydrocapsules, which are capsules with a polymeric outer coating produced in different sizes (Toreki et al., 2004). Hydrocapsules with a diameter of 1−2 mm have been

FIGURE 3.5 *Nesidiocoris tenuis* female ovipositing in an artificial substrate. *Photo: V. De Puysseleyr and T. Machtelinckx.*

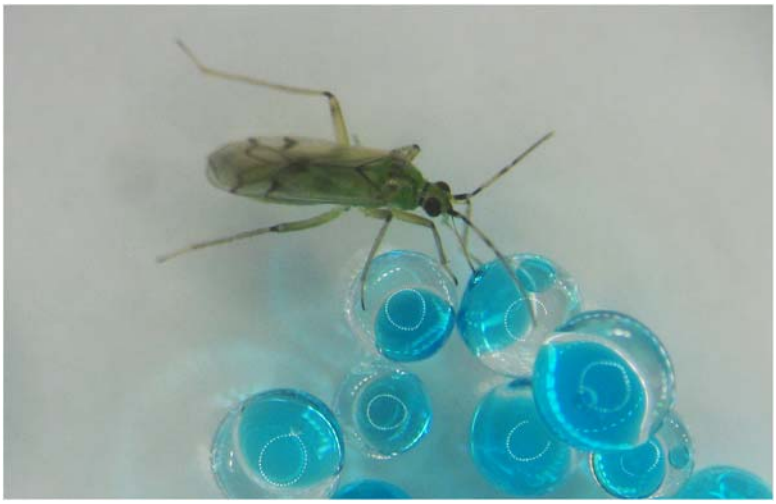

FIGURE 3.6 *Nesidiocoris tenuis* female feeding on a water containing hydrocapsule. *Photo: P. Urbaneja-Bernat.*

used to supply water or sucrose solutions to anthocorid and mirid bugs (Fig. 3.6) (e.g., Shapiro and Ferkovich, 2006; Shapiro et al., 2009; Urbaneja-Bernat et al., 2013).

Most predatory heteropterans used as biological control agents are deployed in natural environments, that is, they forage, feed, mate and rest on plant surfaces. However, plant surfaces are often absent in rearing systems used to produce the predators. Absorbent paper toweling has been widely used as a substitute for a living substrate in culture containers of predatory bugs. When wadded or shredded, the paper toweling increases the surface area and spatial complexity of the rearing environment, providing hiding places for the predators. Furthermore, the paper toweling may allow the absorption of fluids secreted by the predators or their prey (Cohen, 1985; De Clercq et al., 1988). Other paper-based materials, like honeycomb paper and corrugated cardboard strips, have been used to create a living environment for predatory bugs in culture (e.g., Parajulee and Phillips, 1992; Blümel, 1996; Bueno et al., 2006). Absorbent paper materials may not always be an ideal substrate for predatory bugs. Bonte and De Clercq (2010a) demonstrated that survival of *O. laevigatus* on lipophilic surfaces such as wax paper was higher than on absorbent household paper. Waxy paper materials were also used in the rearing of other *Orius* spp. (e.g., Ferkovich and Shapiro, 2004a; Bonte et al., 2012) and of *M. pygmaeus* (Vandekerkhove et al., 2011). Blümel (1996) also reported good results when using vermiculite as a substrate both in culture and storage containers of *Orius* spp. Bueno et al. (2014) tested different substrates for storing and shipping adults of *O. insidiosus* and found that a mixture of vermiculite and rice hulls yielded best survival and poststorage performance.

3.4 Abiotic conditions

3.4.1 Optimal climatic conditions for rearing

Arguably, insects should be reared under environmental conditions (temperature, humidity, light conditions) that allow optimal development and reproduction. Whereas insects in the field are confronted with variable climatic conditions, most rearing is done at approximately constant conditions of temperature, relative humidity and light regime (see Cohen, 2018, for a review). Numerous workers have studied the relationship between temperature and life history parameters of predatory heteropterans, but they usually did so with practical application in biological control programs in mind, rather than considering its implications for mass rearing. For information on the impact of temperature on the bionomics of various families within the Heteroptera we refer to different chapters in Schaefer and Panizzi (2000). Nonetheless, some workers have specifically set out to find the optimal temperature regime for the production process of predatory bugs. Recent examples can be found in Baek et al. (2014), Vacari et al. (2014), and dos Santos et al. (2018) for *Podisus* spp., in Youssef and Abd-Elgayed (2015) for the reduviid *Amphibolus venator* Klug, in Martinez-Garcia et al. (2016,2017) for the mirids *N. tenuis* and *M. pygmaeus* and in Sobhy et al. (2014) and Ding et al. (2016) for the anthocorids *Blaptostethus pallescens* Poppius and *Orius minutus* (L.), respectively.

Less attention has been given in the literature to the effects of relative humidity and light conditions on the performance of heteropteran predators in a mass-rearing system. Overall, humidity extremes are avoided, as low relative humidities may lead to direct dehydration of the insects, and high humidities may result in contamination with fungi or mites (like *Tyrophagus* sp.). The effects of light conditions can be separated into three broad categories: photoperiod, light intensity and light quality (i.e., wavelength) (Cohen, 2018). As a general rule, day-active insects from temperate regions are usually kept at long day conditions (16 hours light and 8 hours dark) to prevent diapause, whereas those originating from warmer/tropical areas can be maintained at shorter photoperiods (see Section 3.VIII for examples). With different types of lighting becoming more widely available, the effect of light quality on insect performance is now receiving increasing interest, but studies on heteropteran predators are few and mostly focus on consequences for the practical use of the bugs as biological control agents under short day conditions in greenhouse crops. Stack and Drummond (1997) found that 9 hours of broad-spectrum light produced by fluorescent lamps supplemented with 6 hours of blue lights prevented diapause of *O. insidiosus* without having adverse effects on the predator's reproduction and nymphal development. Wang et al. (2013) tested development, reproduction and locomotor activity of *O. sauteri* under five light intensities and five wavelengths. They found that at lower light intensities the nymphal period and preoviposition period were extended and fecundity was reduced, whereas egg fertility was improved. Red and blue light slowed down nymphal development and negatively affected preoviposition period, fecundity and egg fertility. Nissinen et al. (2017) assessed the effect of light quality on *M. pygmaeus* by producing a 16L:8D h photoperiod with fluorescent lamps (FLs), high-pressure sodium lamps (HPSLs) and HPSLs complemented with red light-emitting diodes (LEDs). They also tested different photoperiods produced by either FLs or HPSLs. The effect of light quality (FLs vs HPSLs) on fecundity was not consistent throughout the different experiments performed in the study, but the study did show that replacing part of the light produced by HPSL with that of red LEDs did not affect the predator's reproductive biology. Interestingly, the study also indicated that a long photoperiod is not ideal for the reproduction of *M. pygmaeus*, a mirid widely distributed throughout the Palearctic region, which was attributed to the fact that this predator is (partly) night active. This finding may not only be relevant for its practical use in greenhouses, but also for its mass rearing. Optimal climatic conditions for several species of heteropteran predators are listed in papers describing rearing systems in Section 3.VIII.

3.4.2 Cold storage

Several studies have explored the possibilities for cold storage of certain life stages of predatory bugs, as this extends their shelf life and thus allows producers to balance end user demand and product supply (Rathee and Ram, 2018; see also Chapter 2, 2.4.2). Considerable work has been done on the cold storage of predatory pentatomids. De Clercq and Degheele (1993a) stored eggs and adults of *P. nigrispinus* and adults of *P. maculiventris* in complete darkness at 4°C and 9°C and relative humidities (RH) between 50% and 95%−100%. Both temperature and humidity during storage proved to be critical factors, with the lower temperature (4°C) and RH (50%) resulting in poorer survival of eggs and adults. Survival of eggs was also affected by egg age: survival of newly deposited eggs (< 24 hours old) was low even for short storage periods. At 9°C and a RH near 100%, 1- to 4-day-old eggs of *P. nigrispinus* could be kept up to 6 days without adverse effects on viability. At 9°C and 75% RH, adults of both *P. nigrispinus* and *P. maculiventris* could

be stored for up to 1 month without significant mortality, but whereas the former species did not suffer any negative effects on poststorage longevity and reproduction, the latter did. Thorpe and Aldrich (2004) stored *P. maculiventris* for 5 weeks at 5°C or 15°C and either fed them beans and yellow mealworms or starved them during the storage period. Unfed predators suffered greater mortality than their fed counterparts and overall adult survival was better at 5°C than at 15°C. Photoperiod (12 vs 0 hour of light) during storage had no effect. Vacari et al. (2014) and da Costa et al. (2016) found that 15°C was the optimal temperature to store fresh eggs (<24 hours old) of *P. nigrispinus:* the eggs could be stored for 15−17 days without ill effects on poststorage life table parameters. Considering third-instar nymphs of *P. bioculatus* as the ideal stage for release against Colorado potato beetle infestations, de Ladurantaye et al. (2010) investigated short-term storage of second instars of the predator. Their results showed that storage temperatures within the range of 9°C to 15°C and photoperiod (16 or 0 hour of light) did not significantly affect the survival and poststorage development of *P. bioculatus* nymphs stored for periods of 2 to 8 days.

Coudron et al. (2007) evaluated the influence of nutrient quality on the response of eggs, nymphs and adults of *P. maculiventris* when exposed to long-term storage at 4°C or 10°C. Like in De Clercq and Degheele (1993a), survival of all life stages was better at 10°C than at 4°C and adults could be stored for longer periods than eggs (or nymphs). There was a notable effect of diet on the survival of the different stages of *P. maculiventris* during cold storage: whereas eggs and nymphs from a cohort fed on an artificial diet survived storage better than those given live prey, the result was the opposite for adults. The study demonstrated that an optimal food for continuous rearing is not necessarily an optimal food for cold storage. Li et al. (2019) assessed the effect of diet on biological and biochemical parameters of another pentatomid, *A. chinensis*. Survival and longevity after 30-day cold storage, and fecundity after 40-day cold storage were significantly higher for adults reared on pupae of *T. molitor* compared to those given pupae of *A. pernyi*. Adults reared on mealworm pupae had lower supercooling and freezing points, and a higher content of carbohydrates, glycerol and unsaturated fatty acids, indicating that diet affected the accumulation of cold-resistant substances, metabolism level and activities of related enzymes in *A. chinensis* in a manner that enhanced its tolerance to cold storage.

The potential of cold storage was also investigated for anthocorid bugs. Murai et al. (2001) reported that germinated broad bean seeds containing eggs of *O. sauteri* could be stored at 2.5°C−12.5°C for one week without adverse effects on egg hatch. Adults of *O. laevigatus* could be stored for up to 36 days at 10°C with 70% survival and no quality loss in terms of poststorage fecundity (Kim et al., 2009). Bueno et al. (2014) could store *O. insidiosus* for up to 10 days at 8°C without loss of quality. They found that storage of mated females resulted in a markedly higher poststorage fecundity than storage of virgin females. Diapause can provide an effective natural mechanism for the long-term storage of beneficial insects, but little study has been done on predatory heteropterans. Ruberson et al. (1998) reported that adults of *O. insidiosus* showed good longevity and reproduction after storage in diapause for up to 8 weeks at 15°C and a 10 hours photoperiod. Ünal Bahşi and Tunç (2014) stored diapausing adults of *O. majusculus* for 1 month at 18°C and a day length of 11.5 hours, meanwhile providing them with bean pods and *E. kuehniella* eggs; survival rates and fecundity after storage were similar to those reported for nondiapausing adults.

3.5 Crowding and cannibalism

Another important consideration for the production of predatory bugs is rearing density. Crowding leads to a higher degree of competition for food and space and increased stress, which in turn may lead to lower developmental or reproductive success and eventually result in mortality, which may or may not be due to cannibalism. In populations with overlapping life stages, cannibalism is common where older or larger predators prey upon younger and smaller conspecifics (including eggs) (Fig. 3.7) (Arbogast, 1979; Tommasini et al., 2002; Rudolf, 2007; Bonte and De Clercq, 2011; Bueno and van Lenteren, 2012). Age synchronization is therefore an important first measure to reduce cannibalism in mass cultures of predatory bugs (De Clercq and Degheele, 1993c; van den Meiracker, 1999; Bueno and van Lenteren, 2012). Cannibalism can also be avoided to some extent by lowering rearing density in each subsequent developmental stage (i.e., less individuals per container) or by providing more refugia in the rearing arena, using plant materials or artificial substrates. Whereas some studies have reported a reduction of cannibalism in predatory bugs with increasing levels of food supply (Arbogast, 1979; Grundy et al., 2000; Tommasini et al., 2002; Arvaniti et al., 2019), cannibalism has been observed even when nutritionally adequate food is abundant. Bonte and De Clercq (2011) reported that providing ad libitum *E. kuehniella* eggs did not prevent cannibalistic behavior at high nymphal densities in *O. laevigatus*. Higher attractiveness of the mobile conspecific food versus the immobile heterospecific (factitious) food was believed to be the reason for this behavior in this and other species of predatory bugs (Grundy et al., 2000; Bonte and De Clercq, 2011). Whereas some studies have noted higher levels of cannibalism on nutritionally suboptimal food sources like artificial diets (e.g., Cohen, 1985; De Clercq and Degheele, 1992, 1993c), other studies have not observed a clear

FIGURE 3.7 Cannibalism in *Macrolophus pygmaeus*. *Photo: V. De Puysseleyr and B. Vandekerkhove.*

relationship between diet quality and cannibalism in predatory bugs (Leon-Beck and Coll, 2007; Bonte and De Clercq, 2011). Bueno and van Lenteren (2012) concluded that cannibalism is usually not a serious obstacle to mass culturing predatory heteropterans when food and moisture are available.

3.6 Microorganisms

There is very little published information on the impact of microorganisms on the performance of rearing systems for heteropteran predators. Entomopathogenic fungi of the genus *Entomophthora* may cause problems in the production and application of *Macrolophus* bugs (e.g., Nedstam, 2002). However, effective humidity control is sufficient to prevent most fungal problems in the production line (J. Klapwijk, Koppert BV, personal communication).

There is an increase in research on the role of bacterial endosymbionts in beneficial insects. An extensive survey of the presence of *Wolbachia* in 134 species of Japanese terrestrial heteropteran bugs revealed a high infection frequency of approximately 35%, including nabid and lygaeid predators (Kikuchi and Fukatsu, 2003). Machtelinckx et al. (2012) reported that *M. pygmaeus* and *M. caliginosus* were infected with *Wolbachia* and *Rickettsia*. Watanabe et al. (2011) reported that the anthocorid *O. strigicollis* had two strains of cytoplasmic incompatibility (CI) inducing *Wolbachia*.

Wolbachia was responsible for strong CI-effects in *M. pygmaeus* (Machtelinckx et al., 2009). Removal of the endosymbionts in *M. pygmaeus* was, however, not associated with significant fitness effects (Machtelinckx et al., 2012).

The presence of CI-inducing endosymbionts may have important practical implications for the production of these predatory bugs: incompatibility may lead to a strong suppression of population growth when groups with a different infection status interact. This may happen at the production level when a mass culture is infused with insect material of different infection status, or in the field, when growers release predators from different (commercial) sources in their crop. Machtelinckx et al. (2009) concluded that a survey for the presence of *Wolbachia* in commercial and wild populations of economically important predatory heteropterans is therefore highly warranted.

When establishing a colony of predatory heteropterans or infusing it with field-collected insects to increase genetic heterogeneity, it is imperative to check the health status of the feral material. Colonies of predatory bugs face relatively few problems with diseases, but sanitation procedures should be part of the rearing routine. Besides paying attention to entomopathogens, it may be wise to check for the presence of CI-inducing endosymbionts. Finally, a more profound knowledge of the gut microbiota may assist in optimizing foods for production of predatory heteropterans (see Chapter 8).

3.7 Breeding and colony maintenance

To date, the production of heteropteran predators has focused on producing mass numbers wherein the quality, or fitness is as close as possible to that found in nature. Selection for improved performance and reduced production costs would be most desirable and improving host ranges may be attainable. However, production traits are likely to be complex, multigene traits. Information on genes underpinning these desirable traits is lacking. This will require knowledge

of the genetic diversity, gene sequence, and developing phenotypic and genetic markers that identify traits associated with specialized lines.

The study by De Clercq et al. (1998b) indicated heritable genetic variation in developmental rate and body size of *P. maculiventris*. Selection for faster development was useful for improving cost-effectiveness of production, but selection for larger body size was less rewarding because body size was unrelated to fecundity or predation capacity. Mohaghegh-Neyshabouri et al. (1996) observed a lack of correlation between the body weight of females and their fecundity in *P. maculiventris* and *P. nigrispinus*.

Amplified fragment-length polymorphism (AFLP), used to assess genetic variability within or between populations, showed substantial variability within a field population of *P. maculiventris*, which suggests an opportunity for trait selection (Kneeland et al., 2012). A significant amount of the genetic variability found in the field population was retained after it had been maintained in the laboratory for over 100 generations, resulting in little variability between the field and laboratory populations. Retention of genetic variability after extended laboratory rearing provides gene-based evidence for retention of fitness attributes (such as fecundity, longevity and predation capacity) in mass-produced predators. The greatest variability was between populations from different geographical locations (Kneeland et al., 2012). These results indicate that sufficient genetic variation does exist within a field or laboratory population to support breeding efforts for this heteropteran predator and that crosses among populations may provide additional opportunities to enhance breeding efforts.

Knowledge of the changes in gene expression associated with specific traits can assist in breeding programs. For example, a *Drosophila* cDNA microarray comparison of gene expression between adult females of the heteropteran predator *P. bioculatus* identified 6 genes associated with decreased fecundity and one gene associated with increased fecundity (Coudron et al., 2006). The most pronounced differentially expressed gene was actin, which was upregulated in low fecund females. Other upregulated genes in low fecund females included a protein kinase, transcription factor and several genes involved in cellular development. The one upregulated gene in high fecund females was an RNA processing gene, which is consistent with the finding that high fecund females had nearly twice the amount of RNA. Perhaps molecular markers such as these will serve as early indicators of positive or negative responses that correlate with physiological traits such as fecundity and used to direct and accelerate breeding programs.

Mackauer (1976) recommends that one should initiate a colony with a sufficiently high number of founders and maintain a minimum population level to limit the effects of inbreeding or genetic drift. Castañé et al. (2014) investigated the effect of founder population size on the life table parameters and predation capacity of *O. laevigatus* after 5 and 10 generations of laboratory rearing on *E. kuehniella* eggs. Laboratory lines were started from 1, 10, or 50 founder couples from field-collected adults. The 1 founder couple line performed worse than the 10 and 50 founder couples lines in all parameters. Whereas the 10 and 50 founder couples lines performed better with time, no such adaptation to the laboratory environment was seen in the 1 founder couple line. Overall, no difference was found between the 10 and 50 founder couples lines, suggesting that colonies of *O. laevigatus* may be started from as few as 10 founder couples without significant loss of quality.

De Clercq et al. (1998b) studied the effect of inbreeding in the pentatomid predator *P. maculiventris* throughout 30 generations following introduction in the laboratory. There were consistent differences in body size among two inbred lines (started from a single couple) and a reference line in most generations, but fecundity did not differ among populations. Developmental, reproductive and behavioral parameters indicated no deleterious effects of inbreeding. In generation 30, fecundity of all lines had dropped to about half of that in generation 15, which was attributed to nongenetic factors.

Mohaghegh et al. (1998a,b) studied the effects of maternal age and egg weight on offspring performance of *P. maculiventris* and *P. nigrispinus*. They observed that in *P. maculiventris* offspring from large eggs developed faster, weighed more and in turn yielded larger eggs than offspring from small eggs. Further, they also found that in both species offspring of young parents took less time to complete development and achieved heavier body weights compared to offspring of old parents. Consequently, these researchers recommended the use of young parents (2−4 weeks old) for egg production.

Bonte and De Clercq (2010c) found that the age of males influenced the reproductive output of *O. laevigatus* females: on nutritionally suboptimal diets, mating with newly emerged males resulted in slower oocyte development. On such suboptimal foods, they advised placing female adults together with sufficient numbers of males older than 5 days to ensure successful mating. Leon-Beck and Coll (2009), on the other hand, suggest removing all males from rearing within 2−3 days after male emergence, as females maintained with males have lower fecundities than those without males for the remainder of the oviposition period. Based on similar findings, Torres et al. (2006) recommend maintaining female-biased sex ratios in colonies of *P. nigrispinus* to reduce the costs of male maintenance.

3.8 Mass-rearing systems

For reasons of confidentiality, there is little published information on commercial mass-rearing systems for predatory heteropterans. However, several papers have described systems for medium- to large-scale production of economically important predatory heteropterans. The procedures described in a selection of exemplary studies are summarized below. Major factors which were indicated to determine the success of a rearing system were climate, food quality and quantity, living and oviposition substrate, type of container and rearing density.

Schmidt et al. (1995) produced nymphs and adults of *O. insidiosus* at 24°C, 70% RH and a 16L:8D h photoperiod in 4.5L zip-lock plastic bags, containing 200−600 individuals of similar age. The insects remained in the same bag from egg to reproducing adults. Eggs of both *S. cerealella* and *E. kuehniella* were offered to the bugs coated on moistened cardboard strips. Green bean pods and stems were placed in the bags to provide water and oviposition sites. Excess condensation was removed with absorbent material. Blümel (1996) reared *O. laevigatus* and *O. majusculus* at 25°C, 70% RH and a 16 hours photophase on frozen *E. kuehniella* eggs in polypropylene boxes, with corrugated board scrapes. The latter author used cyclamen leaves as an oviposition substrate. Tommasini et al. (2004) describe a rearing procedure for *O. laevigatus* at 26°C, 75% RH and a 16L:8D h photoperiod. The procedure started by placing about 1500 eggs deposited in green beans in 3.6L plastic boxes furnished with buckwheat hulls. Nymphs and adults were fed twice per week with (frozen) *E. kuehniella* eggs glued on paper strips. The whole life cycle was completed in the same container. Adults were kept for oviposition for about 4 weeks and bean pods were changed twice per week. Ito (2007) developed a simple method to mass rear *O. strigicollis* and *O. laevigatus* at 22°C, 45% RH and 16 hours of light in petri dishes (9 cm diameter, 2 cm high) containing 30 g of heat-sterilized wheat grains, using (frozen) *E. kuehniella* eggs as food and a fresh soybean seedling as a water supply and oviposition substrate. This method was used to produce both species at initial nymphal densities of 400 individuals per petri dish. The insects were easily separated from the wheat grains using a sieve. Bueno et al. (2006) and Bueno and van Lenteren (2012) provide descriptions of rearing systems for *O. insidiosus*. Bueno et al. (2006) tested plastic bags (4 L), petri dishes (0.8 L) and glass jars (1.7 L) as containers at different rearing densities and a mean temperature of 26°C, 70% RH and a 12 hours photophase. They found that both the immature and adult stages could be reared in glass jars, using *E. kuehniella* eggs as food and farmer's friend inflorescences as moisture source and oviposition substrate. Diaz et al. (2020) described a batch production system to rear *O. insidiosus* and studied the effect of food, environmental conditions, and maintenance frequency on development and reproduction of the anthocorid in the rearing system. A mixed diet, consisting of *S. cerealella* eggs and *F. occidentalis* thrips, allowed an 8.4 times greater production of new adults than when a single food source was used. Constant laboratory conditions (24°C ± 1°C) resulted in 4.9 times greater adult production than a greenhouse environment (17.9°C ± 5°C). Interestingly, a 48-hour feeding frequency produced 4.2 times more *O. insidiosus* adults than a 24-hour frequency. The amount of lepidopteran eggs to be supplied to nymphal and adult containers was based on a study by Yano et al. (2002), who calculated the minimum number of eggs needed per individual for optimal development and reproduction of the related (but smaller) anthocorid *O. sauteri*. Further improvements of this rearing method allowed the production of ca. 8000 adults per glass jar from an initial 250 eggs (Bueno and van Lenteren, 2012). Thomas et al. (2012) described the mass rearing of *Orius pumilio* in 6.4 L containers with buckwheat hulls, fed *E. kuehniella* eggs and Hydrocapsules containing water at 25°C, 75%−80% RH and a 14 L:10D h photoperiod; green beans were used as a substrate for oviposition. New colonies were started weekly with approximately 18,000 eggs. Based on oviposition data and the analysis of yolk protein accumulation by ELISA, they recommended that the maximum period to maintain an adult colony for egg production should be about 1 month.

Grundy et al. (2000) presented a mass-rearing method for the assassin bug *P. plagipennis*. When reared at 25°C, 50%−70% RH and a 15 L:9D h photoperiod on a diet of heat-killed yellow mealworm, *T. molitor*, larvae, the optimum rearing density was 20−27 nymphs per 5 L container, based on an assessment of nymphal mortality, developmental time and adult body weight. The optimum density for oviposition was 16 adults per 5 L container. Fecundity was better on a mixed diet of *T. molitor* and *H. armigera* than on *T. molitor* alone.

Rearing methods for *Podisus* bugs were described by De Clercq et al. (1988), De Clercq and Degheele (1993c) and Torres et al. (2006). The latter authors provided a description of mass production methods for *P. nigrispinus* in Brazil. Cage designs for nymphs and adults were described in detail and illustrated. Up to 600 nymphs could be raised in 11 L plastic trays. Adults were kept in 8 L Plexiglas cages at a density of 18 individuals per cage. In order to reduce costs for the maintenance of males, a 2:1 female: male sex ratio was maintained in the adult cages. Several natural and factitious prey were used but the best results, also in terms of cost-efficiency, were obtained with mealworms and housefly larvae. Rearing methods for these factitious prey were also presented.

3.9 Conclusion

Heteropteran predators probe and feed on a wide variety of insects, plants and organic matter in nature; some possibly are omnivorous, some polyphagous, and some are specialists (Torres and Boyd, 2009). Our ability to rear these insects in small or large numbers varies from species to species for reasons that remain largely unknown. This chapter highlights the progression of efforts to rear heteropteran predators and demonstrates that researchers have amassed a large amount of information, ranging from feeding behavior to the chemical make-up of natural and artificial foods. Most information is available for those insects that are effective predators or successfully reared.

The performance of alternative food sources has been compared to the insect's natural prey with respect to growth and other commonly accepted fitness parameters. Life history parameters are the most common types of available information and in contrast, genetic information is relatively rare. Not all information is proving to be as useful as first thought. For example, several inconsistencies were found when associating increased weight and size with improved fecundity or fitness. By comparison, some information with seemingly high correlative value is rare. For example, ovarian development is an indicator of dietary effect and the chemical composition of the predator to aid in diet formulation. Consequently, these parameters may have been underutilized. In all cases, we should measure nutritional parameters over several generations to detect adaptation or selection trends as well as chronic effects only detectable after prolonged feeding.

Most heteropteran predators are reared on natural or factitious prey. Yet the lure of an artificial diet remains because of desired advantages and because of numerous successes of pest insects reared on artificial diets, including some heteropteran species (e.g., *Lygus* spp.). Although several heteropteran predators have been reared for consecutive generations on artificial diets devoid of living material, no predatory heteroperans are currently mass-reared commercially on an artificial diet.

Arguably, we are missing important knowledge of genetics, behavior, and developmental and reproductive biology in the case of heteropteran predators that would assist our production efforts on factitious prey as well as artificial food. A case could also be made for shortfalls in nutritional information, for example, information about trace nutrients. Optimizing artificial oviposition substrates for predatory bugs will require a more thorough understanding of their reproductive ecology, with specific details of their oviposition behavior (Lundgren, 2011). Likewise, the microbiota in heteropterans is important, but analysis, preservation and manipulation of microbiota have eluded researchers. Each of these areas represents future opportunities for improving and expanding our ability to produce heteropteran predators.

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Chapter 4

Production of dipteran parasitoids

Maria Luisa Dindo[1] and Simon Grenier[2]
[1]Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy, [2]Rue des Mésanges, Chassieu, France

4.1 Introduction

Dipteran parasitoids are underestimated and often forgotten regarding their role in biological control. This is partially due to their relatively low number, as they represent about 20% of all insect parasitoids, the majority of which are hymenopterans (Feener and Brown, 1997). Nevertheless, they constitute the second most important group of parasitoid insects, due to their biological, physiological and behavioral characteristics, which include their various parasitization modes that allow them to take possession of the hosts in different environments and thus reduce insect pest populations. Many representatives of this group (mainly Tachinidae and Bombyliidae) are unique, because of their ability to attack hosts hidden in vegetables or in the soil (Mellini, 1997).

Dipteran parasitoids are far less studied than parasitic wasps from all points of view, for example, biology, the host−parasitoid relationship, and also applied biocontrol. Yet, the research performed has shown that they play a major role in regulating phytophagous insect populations, and their importance is increasingly recognized, especially in natural biological control (Cutler et al., 2015; Camerini et al., 2016; Moore et al., 2019; Rodríguez et al., 2019). The general image of "flies," however, remains unpopular.

Compared to hymenopteran parasitoids, far less research has been performed on dipterans, including the development of rearing technology, and, to date, "mass" production has been performed for only a few species. Moreover, their large size relative to most of the wasp parasitoids is unfavorable for mass production, which increases the need for food and space to obtain a production level comparable to what has been achieved with hymenopterans. Yet, several studies concerning the rearing of these beneficial insects have been carried out. The purpose of this chapter is to review the work done with some new perspectives and bring this group of parasitoids to light.

Eggleton and Belshaw (1992) acknowledged parasitoids in many families of Diptera, but only a few (i.e., Tachinidae, Sarcophagidae, Phoridae, Chryptochaetidae and Bombyliidae) include species of interest for applied biological control that would be also of interest for production. This chapter will be mainly (but not exclusively) devoted to the Tachinidae, which comprise about 8500 species described worldwide (O'Hara, 2013), and represent the largest and most important family of nonhymenopteran parasitoids. Some examples of tachinids and other dipteran parasitoids implicated in biological control will be presented in the first section. Subsequently, the most important aspects of their biology relevant for rearing will be described; then, production techniques, in vivo as well as in vitro, from different points of view will be considered. Finally, some perspectives with the aim of stimulating new ideas and new research efforts on these topics will be presented.

4.2 Dipteran parasitoids as biocontrol agents

We will briefly summarize some examples of the use of dipteran parasitoids in classical and augmentative biocontrol programs (sensu van Lenteren, 2012), as the success of these types of biological control (especially augmentative) is widely based on the development of efficient techniques for culturing the entomophagous species involved (Greathead, 1986). Several species have shown the potential to control target insect pests, but fewer have been utilized in biocontrol programs. According to recent available information, some of these dipteran parasitoids have been or are mass cultured in state-funded facilities (still in Cuba, as discussed in this chapter), but production in commercial biofactories has only very rarely occurred. On the contrary, dipteran predators, such as *Aphidoletes aphidimyza* Rondani (Cecidomyiidae)

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and *Episyrphus balteatus* (De Geer) (Syrphidae), have been marketed and used as biocontrol agents (van Lenteren, 2012). Fly parasitoids could potentially be better exploited in biological control if the technology for their commercial production is developed. Examples of dipteran parasitoids utilized in applied biological control programs are presented in Table 4.1.

4.2.1 Tachinidae

There are many examples of attempts (either successful or not) to utilize Tachinidae in classical and augmentative biological control programs, especially in the Nearctic and Neotropical regions and, to a lesser extent, in the Australian and Oceanian regions. It is interesting to note that examples in the Palearctic region are almost completely absent. One of the few attempts in Europe occurred in 1967, when the north American tachinid larval parasitoid *Myiopharus doryphorae* (Riley) was introduced into Poland for the control of *Leptinotarsa decemlineata* (Say). The parasitoid became established, but information about its impact on the target host species is lacking (Gerber and Schaffner, 2016).

The results achieved by these programs until the late 1980s were reviewed by Grenier (1988a), who has also analyzed the reasons for successes and failures. Failures were often related to a lack of knowledge regarding the biology, behavior, and ecology of the species used as well as improper rearing techniques and shipment procedures. Grenier (1988a) reported different examples of classical and augmentative biological control programs performed against forest lepidopterous defoliators and sawflies in North America using tachinid flies. One of the best successes was achieved with *Cyzenis albicans* Fallen, which was imported from Europe and released in Canada (15,000 flies in 1979−80) to control the winter moth *Operophtera brumata* L. The fly established and helped to control the target insect pest over several years (Hulme and Green, 1984; Horgan et al., 1999). In the early 1900s, 16 tachinid species were introduced from Europe to the northern United States (US) (New England) to control the gypsy moth *Lymantria dispar* (L.) and the brown tail moth *Nygmia phaerrhoea* Don. Two species—namely *Blepharipa pratensis* Meigen and *Compsilura concinnata* Meigen—became established in many US states and were later used for augmentative releases of 83,658 flies against the gypsy moth (Blumenthal et al., 1979). Two other species, *Exorista larvarum* (L.) and *Parasetigena silvestris* (Robineau-Desvoidy), well known as antagonists of *L. dispar* in Europe, were used in inoculative releases in the northern US and became established (Sabrosky and Reardon, 1976; Kenis and Lopez Vaamonde, 1998). At the laboratory of entomology of the University of Bologna, *E. larvarum*, has also been the subject of extensive research concerning mass production, especially on artificial media, as reported further in this chapter.

Another tachinid of European origin, *Aphantorhaphopsis samarensis* (Villeneuve), is considered a good candidate for biological control of the gypsy moth due to its limited host range, which may result in a lower risk to nontarget lepidopterous species (Fuester et al., 2001). This tachinid was repeatedly introduced in the northern United States and Canada, but its establishment has not been confirmed (McManus and Csóka, 2007). Similarly, *Lydella jalisco* Woodley, a parasitoid of Mexican origin, was repeatedly introduced from its native Mexico into Texas for the classical control of the Mexican rice borer, *Eoreuma loftini* (Dyar) (a key pest of sugarcane in Texas), with limited results (Lauzière et al., 2001). Conversely, *Lixophaga diatraeae* Townsend, originating from Cuba, is one of the species more effectively utilized, in both classical and augmentative biological control, against lepidopterous sugarcane borers of the genera *Diatraea* and *Chilo*, especially *Diatraea saccharalis* (F.). As reviewed by Grenier (1988a), between 1915 and 1971 *L. diatraeae* was extensively introduced into different sugarcane-producing countries (e.g., in the southern United States, West Indies islands, South America, and Southeast Asia) and established in some of these regions (including Louisiana, Guadalupe, St. Kitts, Colombia, and Brazil). The successful establishment was not dependent on the number of flies released but rather on the climatic and ecological conditions in the countries of introduction. Usually, the best successes were obtained in islands. Grenier (1988a) also reported various examples of effective control of *D. saccharalis* populations following augmentative releases of *L. diatraeae* in different areas of introduction, including Louisiana and Florida. These results were achieved despite the high mobility of the flies and the continental position of these states (King et al., 1981). Augmentative releases of *L. diatraeae* to control *D. saccharalis* and other lepidopterous sugarcane borers have been (and are currently) widely and successfully carried out in Cuba, which is the country of origin of the tachinid (Scaramuzza, 1951; Nicholls et al., 2002; Montes, 2008; Grillo Ravelo et al., 2015). The control of *D. saccharalis* is one of the main objectives of Cuba's National Biological Control Program, which was created by the Sugar Ministry (MINAZ) in 1980 and is still in progress in large areas of the country (Perez-Consuegra et al., 2018; Marquez et al., 2020). Medina (2002) reported that, inside this program, more than 100 million *L. diatraeae* flies were produced per year in a network of 54 reproduction centers of entomophagous insects and entomopathogens (CREE = Centros Reproductores Entomófagos y Entomopatógenos) of entomophagous insects and entomopathogens. They were established in all 15 provinces of the island (Massó Villalón, 2007). Fly releases over 1.6 million ha have led to successful

TABLE 4.1 Examples of dipteran parasitoids utilized in classical (C) or augmentative (A) biological control programs and described in the text.[a]

Parasitoid				
Family	**Species**	**Target insect**	**Type of biological control**	**Country of application**
Tachinidae	*Myiopharus doryphorae*	*Leptinotarsa decemlineata*	C	Poland
	Cyzenis albicans	*Operophtera brumata*	C	Canada
	Aphantorhaphopsis samarensis	*Lymantria dispar*	C	Canada and Northern United States
	Exorista larvarum			Northern United States
	Parasetigena silvestris			
	Blepharipa pratensis	*Lymantra dispar* and *Nygmia phaerrhoea*	C and A	
	Compsilura concinnata			
	Lydella jalisco	*Eoreuma loftini*	C	Southern United States (Texas)
	Archytas marmoratus	*Helicoverpa zea* *Spodoptera frugiperda*	A[b]	Southern United States
	Lixadmontia franki	*Metamasius callizona*	C	Southern United States (Florida)
	Ormia depleta	*Scapteriscus* spp	C	
	Lydella minense *Billaea claripalpis*	*Diatraea* spp.	C and A	Brazil, Colombia and Peru
	Lixophaga diatraeae	*Diatraea saccharalis* and other lepidopterous sugarcane borers	C and A A	Southern United States, West Indies Islands, South America, and Southeast Asia Cuba
		Chilo infuscatellus and other lepidopterous sugarcane borers	C and A[b]	China (Guangxi)
	Trichopoda pennipes	*Nezara viridula*	C	Different countries
	Trichopoda giacomelli		C	Australia
	Bessa remota	*Levuana iridescen*	C	Fiji islands
Sarcophagidae	*Agria housei*	*Choristoneura fumiferana*	C	Canada
Cryptochaetidae	*Cryptochaetum iceryae*	*Icerya purchasi*	C	United States (California), Mauritius and Israel
	Cryptochaetum jorgepastori	*Paleococcus fuscipennis*	C	Israel
Phoridae	*Pseudacteon* spp.	*Solenopsis* spp.	C	Southern United States

[a] *There are also additional examples in different regions of the world.*
[b] *At experimental level.*

biocontrol of *D. saccharalis*. Moreover, *L. diatraeae* was also imported into China (Guangxi) to control *Chilo infuscatellus* Snellen and other sugarcane lepidopterous pests; high levels of efficacy in controlling these target insects were observed in laboratory and field trials (Deng et al., 2008, 2010).

In Latin America, other tachinids, including *Lydella minense* Townsend from Brazil and *Billaea claripalpis* (van de Wulp) from Mexico, have been utilized against sugarcane borers of the genus *Diatraea*, in either classical or augmentative biological control programs (Grenier, 1988a; Weir et al., 2007; Bustillo Pardey, 2009; Parra, 2014). Arrigoni (1992) reports that in the 1980s, 5.7 million flies of both species were produced in 17 facilities of Copersucar, which is the biggest Brazilian sugar and ethanol company. *L. minense* has also been produced by a commercial biofactory in Colombia (Laboratorios Biocol, La Victoria Valle) and, similarly to *B. claripalpis*, it is currently used for the biological control of sugarcane borers (Vargas et al., 2015; Kondo et al., 2020).

During the 1980s and 1990s in the southern United States, *Archytas marmoratus* (Townsend), a native larval−pupal parasitoid of noctuids, was successfully mass produced and used in augmentative field release experiments against the corn earworm *Helicoverpa zea* (Boddie) and the fall armyworm *Spodoptera frugiperda* (J. E. Smith) in whorl-stage corn (Gross, 1990a; Proshold et al., 1998). The parasitoid releases were also combined with other techniques such as inherited sterility (Mannion et al., 1995). Two other tachinids, *Eucelatoria bryani* Sabrosky and *Palexorista laxa* Curran, were used in field cage release experiments against *Heliothis virescens* (F.) and showed potential for controlling this target pest (King and Coleman, 1989). However, despite these promising results, further investigation or implementation of biocontrol using *A. marmoratus*, *E. bryani*, and *P. laxa* was apparently not pursued.

Trichopoda pennipes (F.), which is native to the southern United States and South America, is a parasitoid of the green vegetable bug *Nezara viridula* (L.) and has been introduced into different countries throughout the world to control the target insect, with mixed success (Grenier, 1988a; O'Hara, 2008). In Italy its introduction, followed by establishment, was fortuitous (Colazza et al., 1996). A few years later, attempts to introduce *Trichopoda giacomelli* (Blanchard) in Australia (Queensland) were successful as the parasitoid has established and proved capable of decreasing populations of *N. viridula* (Coombs and Sands, 2000).

A wide classical biological control program was carried out in Florida with the tachinid *Lixadmontia franki* Wood and Cave being used against the Mexican bromeliad weevil *Metamasius callizona* (Chevrolat), which is a native insect of Mexico and Guatemala. *Lixadmontia franki*, originally found in Honduras on a congeneric species of bromeliad-eating weevil, was imported, mass reared, and repeatedly released (2279 flies in total) throughout 2007−09 in four sites. However, postrelease monitoring has recovered only two F2 flies at one site (Cooper et al., 2011). Among the suggested reasons for the minimal success achieved were the differences in climate and elevation between the areas of the parasitoid's origin and its introduction, and also a lack of knowledge of parasitoid biology and difficulty in rearing, which limited the number of flies available for releases. In Florida, a classical biological control program was also conducted, with more success, against mole crickets of the genus *Scapteriscus* (an exotic pest from South America). A natural enemy, the tachinid *Ormia depleta* (Wiedemann), was imported from Brazil and released at different sites in Florida. The parasitoid became established, spread throughout southern Florida, and effectively contributed to controlling the target insects (Frank et al., 1996; Frank and Parkman, 1999).

4.2.2 Other dipteran parasitoids

Besides tachinids, other dipteran parasitoids have shown potential as biocontrol agents. In 1971, 2800 *Agria housei* Shewell (Sarcophagidae), which were obtained from a laboratory culture, were released (unsuccessfully) in Canada against the spruce budworm *Choristoneura fumiferana* Freeman (Hulme and Green, 1984). Sarcophagid flies of the genus *Blaesoxipha* are known as natural enemies of grasshoppers. In the years 1937−40, they were introduced either into Argentina from Canada [*B. aculeata* (Aldrich), *B. atlantis* (Aldrich), *B. hunteri* (Hough), *B. opifera* (Coquillett), and *B. reversa* (Aldrich)] or into Canada from Argentina [*B. caridei* (Brethes), *B. australis* (Blanch), and *B. neuquenensis* (Blanchard)] for classical biological control. Establishment was reported only for *B. caridei* and *B. neuquenensis* (Clausen, 1962; Greco et al., 2020). Moreover, in the early 1970s attempts to establish *B. filipjevi* Rohdendorf in Barbados from East Africa failed (van Lenteren and Colmenarez, 2020). As shown by Mason and Erlandson (1994) and Danyk et al. (2005) the potential of *Blaesoxipha* parasitoids as biocontrol agents is not well known yet.

Encouraging results have been achieved with Cryptochaetidae, a small family parasitizing Margarodidae. Greathead (1986) reported the successful introduction of two Cryptochaetidae of Australian origin into California and Mauritius to control *Icerya purchasi* Maskell and *Icerya seychellarum* (Westwood), respectively; however, these introductions were later neglected due to the superior performance and ease of handling of *Rodolia cardinalis* Mulsant. In the 1990s, *Cryptochaetum iceryae* (Williston) was introduced from California to Israel for biological control of *I. purchasi*,

suppressing the target insect in 2 years at some release sites (Mendel and Blumberg, 1991). Another species, *Cryptochaetum jorgepastori* Cadalha, was introduced to Israel from Spain and contributed to the control of the homopteran pest, *Palaeococcus fuscipennis* (Burmeister) (Mendel et al., 1998).

Extensive research conducted in the southern US has focused on the potential of introducing phorid species of the genus *Pseudacteon* (from South America) as biocontrol agents of the imported *Solenopsis* fire ants. Some introductions (e.g., six *Pseudacteon* species throughout the southern United States) were successful despite difficulties in rearing the parasitoid (Oi et al., 2015; Porter and Plowes, 2018). The effectiveness of introduced *Pseudacteon* spp. in regulating population densities is, however, doubtful and, especially, difficult to quantify (Morrison, 2012; Chen and Fadamiro, 2018). One of the problems may be the exceedingly high specificity of *Pseudacteon* to their *Solenopsis* host species and even biotype. For example, laboratory studies conducted in the US suggested that *P. bifidus* Brown & Morrison, an antagonist of *S. geminata* (F.), could be effective as a biological control agent of this fire ant (Porter and Plowes, 2018). Yet, in other laboratory studies, *P. bifidus* proved to be highly specific, and not very suitable to the Galapagos *S. geminata* biotype, thus appearing improper to be imported into the archipelago for classical biological control programs against the local fire ant (Castillo et al., 2020). Despite difficulties, however, phorid flies are still believed to be valid biocontrol agents of invasive *Solenopsis* ants (Chen and Morrison, 2020).

Although they are hard to handle in captivity, some bombyliids of the genus *Exhyalanthrax* could be promising biological control agents against *Glossina* spp. and merit further consideration (Greathead, 1980). In special habitats, *Villa cana* (Meigen) appeared to regulate some populations of *Thaumetopoea pityocampa* (Denis and Schiffermuller) (Biliotti et al., 1965).

4.2.3 Side effects

As it is well known, classical biological control has raised some concern about the potential negative effects of exotic natural enemies, mainly polyphagous predators and parasitoids, on indigenous nontarget species (Howarth, 1991). One of the best-known examples is the tachinid *Bessa remota* (Aldrich), which was successfully introduced from Malaysia into the Fiji Islands in the 1920s, where it provided effective control of the coconut moth *Levuana iridescens* Bethune-Baker but was subsequently believed to have caused the extinction of both the target and some nontarget species. Extinction is, however, a controversial issue, since the shortage of reports of *L. iridescens* was mainly due to the declining value of copra, which resulted in less research on coconuts, and a lack of a comprehensive campaign for explorations of coconut palm fronds (Hoddle, 2006). Likewise, *C. concinnata*, which has more than 200 known hosts besides the target species *L. dispar*, has been suspected as causing the decline of indigenous lepidopterans in North America, including giant silk moths (Saturniidae) (Elkinton and Boettner, 2012) and the monarch butterfly *Danaus plexippus* (L) (Oberhauser et al., 2017). However, these concerns about classical biological control do not suggest a need to prohibit the importation of natural enemies. Classical biocontrol remains the best way to control nonintentional introductions of insect pests. Nevertheless, more careful studies on the impact of introductions on indigenous species are needed (van Lenteren et al., 2006; Kenis et al., 2017). Regulations to control the introductions of macroorganisms in many countries are now commonplace (Barratt et al., 2018). The possibility of rearing an exotic parasitoid may enhance one's ability to obtain more knowledge on its biology, host range, and host−parasitoid interaction, thus providing useful information regarding the risks and benefits of natural enemy introductions: also in this view, research concerning the production of parasitoids, including tachinids and other dipterans, is very important and needs to be encouraged.

4.3 Aspects of dipteran parasitoid biology of special interest for production

A deep knowledge of parasitoid biology (especially host range, oviposition strategies, and host−parasitoid interaction) is a key tool to increasing chances of rearing success. More exhaustive information on the biology of fly parasitoids may be found in specialized reviews, such as Clausen (1962) and Feener and Brown (1997) for dipteran parasitoids in general; Mellini (1991), Belshaw (1994), Stireman et al. (2006), and Dindo (2011) for tachinids; Pape (1987) for sarcophagids; Quezada and DeBach (1973) for cryptochaetids (e.g., *C. iceryae*); Disney (1994) for phorids; and Yeates and Greathead (1997) for bombyliids.

4.3.1 Host range

Tachinids parasitize an extensive range of plant-feeding insects. Hosts are primarily larval Lepidoptera, but a variety of insects of other taxa are also hosts, including Heteroptera, Dermaptera, Phasmatodea, Orthoptera (nymphs and adults),

Coleoptera (larvae and adults), and larval Hymenoptera Symphyta (Cerretti and Tschorsnig, 2010; O'Hara et al., 2020). About 70% of the known host species are Lepidoptera, and among them, more than 50% belong to four families of economic importance (Noctuidae, Geometridae, Tortricidae, and Pyralidae) (Grenier and Liljestrhöm, 1991). The hosts of most tachinid species are, however, still unidentified. In 1991, over one-half of the European species of tachinids had no known hosts (Mellini, 1991). No tachinid species are known to parasitize eggs or pupae, although many are larval−pupal parasitoids. Many tachinids are polyphagous (i.e., *E. larvarum* and *C. concinnata*), whereas others have been found to exhibit relatively narrow host ranges (i.e., *T. giacomelli* and *Phryxe caudata* Rondani). In some cases, oviposition occurs preferably on hosts of one sex, males (e.g., *T. pennipes*) or females [(e.g., *Istocheta aldrichi* (Mesnil)] (Grenier and Liljestrhöm, 1991).

Of the families considered in this chapter, sarcophagids are known largely as saprophagous, but some species are parasitoids. Some genera (e.g., *Agria*, *Sarcophaga*, and *Blaesoxipha*) contain parasitoid species of forest or agricultural insect pests (Lepidoptera for *Agria* and *Sarcophaga*, Hymenoptera Symphyta for *Agria*, and Orthoptera and Coleoptera for *Blaesoxipha*) (Pape, 1994). Millipedes as well as nonarthropod invertebrates (e.g., helicid snails and earthworms) are also known as hosts of sarcophagid parasitoids (Pape, 1990; Shoaib and Cagan, 2004; Mello-Patiu, 2016; Brousseau et al., 2020). Cryptochaetids are parasitoids of Homoptera Margarodidae, whereas many phorids are parasitoids of ants. For example, in South America, 22 species of the genus *Pseudacteon* are natural enemies of fire ants (Chen and Porter, 2020). The recorded host range of bombyliids includes seven insect orders and also some Araneae. Almost one-half of the records are from Hymenoptera (bees and wasps), but also tsetse flies and some lepidopterous larvae are attacked, whereas a few species are hyperparasitoids of tachinids (Yeates and Greathead, 1997). A record also exists of a bombyliid attacking the fruit fly *Rhagoletis pomonella* Walsh (Muniz-Reyes et al., 2011).

4.3.2 Oviposition strategies

Knowledge of the oviposition strategy adopted, and of the mechanisms involved in host selection, is critical for the parasitization process and for successful continuous in vivo culture of parasitoids (dipterans in our case). This aspect is also crucial for in vitro production because it influences the way that eggs (or larvae) must be obtained to be placed on the artificial medium. In particular, achieving the goal of continuous in vitro culture without the host requires accurate information on the parasitoid oviposition strategy and factors triggering oviposition to induce direct oviposition on the artificial substrate.

Regarding the dipteran parasitoids considered in this chapter, tachinids may adopt direct or indirect oviposition strategies, depending on the species (Grenier and Liljestrhöm, 1991; Dindo and Nakamura, 2018). In direct strategies, oviposition may occur in diverse modes, for example, eggs may be deposited or projected on the host integument [(e.g., *Exorista japonica* Townsend, *E. larvarum*, or *Carcelia gnava* (Meigen)]. Tachinid parasitoids of the adult stinkbug *Thyanta perditor* (F.), such as *Euthera barbiellini* Bezzi and *Trichopoda* cf. *pictipennis* Bigot, deposit most of their eggs on the dorsal surface of the host body, mainly on the pronotum (Lucini et al., 2020). On other pentatomids, however, eggs are preferably laid on hidden places, for example, the abdominal dorsal surface or even under the wings (Aldrich et al., 2006; Agostinetto et al., 2018). More rarely, eggs are inserted into the host via the genital−anal or buccal cavity [e.g., *Rondania cucullata* Robineau-Desvoidy, a parasitoid of the beet weevil *Conorhynchus mendicus* (Gyllenhal)] or even injected into the host body (e.g., Blondeliini and *E. bryani*). In the latter mode, some sternites of the female are modified as a piercing organ utilized to perforate the host integument and guide the ovipositor, which inserts an egg (or a larva) into the cut. In indirect strategies, females are usually ovoviviparous and the eggs may be placed close to a host. First instars are generally of the planidium type and have to wait for a host to pass by (e.g., *A. marmoratus*) or have to search for a host on their own (e.g., *L. diatraeae*). This latter strategy allows parasitoids to reach hosts that live in concealed places that are inaccessible to adult flies. Most Goniini deposit minute microtype eggs on the host food plant, and these eggs hatch only after being accidentally eaten by a host. About 40% of Palearctic species show indirect modes, but ovipositing on the host body is the most common strategy used by tachinids. Mixed strategies are also possible; for instance, the females of ormiine tachinids may larviposit on or near their hosts (Fowler, 1987).

Sarcophagid flies of the genus *Blaesoxipha* are ovoviviparous or viviparous and may display either direct or indirect oviposition (or larviposition) strategies, similar to those described for tachinids (Allen and Pape, 1996). In Cryptochaetidae, eggs are oviposited directly in the host hemocoel through a "false" piercing ovipositor formed by the terminal segments of the female abdomen. *Pseudacteon* phorids that parasitize fire ants exhibit a direct oviposition strategy, since females hover near hosts and then drop down to insert an egg into the host thorax with a specialized ovipositor (Porter, 1998). The oviposition in bombyliids could be peculiar, thus rendering their rearing very delicate to achieve, as observed in *V. cana* by Biliotti et al. (1965). The female *V. cana* collects sand or dust from the soil in a

special structure (perivaginal "pouch") located at the tip of its abdomen. The female broadcasts its eggs while flying over an area where the host has been detected. The eggs pass through the pouch where they are coated with sand or dust and thus resemble the environment. After hatching, the larva, as a planidium, goes down in the soil and penetrates the pupa by piercing the integument.

The mechanisms of host selection in dipterans are related to their oviposition strategy and rely on chemical and physical stimuli (Nakamura et al., 2013). Many aspects, however, remain unknown as dipteran parasitoids have been far less studied than hymenopterans. In species with direct strategies, females use chemical cues to locate the habitat (plant or other host food sources) and host or cues derived from the interaction between the host and the plant, such as "frass" (Godfray, 1994; Aldrich et al., 2006). For instance, a fire ant alarm pheromone has been shown to attract the phorid fly parasitoid *Pseudacteon tricuspis* Borgmeier (Sharma et al., 2011), whereas host-induced plant volatiles were found to be crucial host localization cues for the tachinids *E. japonica* and *E. larvarum* (Ichiki et al., 2011; Depalo et al., 2012). Physical stimuli, including visual cues, also play a role in the host habitat and host location in dipteran parasitoids, especially tachinids (Stireman, 2002). Host size, color, texture, and movements can affect the oviposition behavior of a number of dipteran parasitoids, including *E. japonica* (Ichiki et al., 2011).

Host location by phonotaxis has been demonstrated for sarcophagids of the genus *Emblemasoma* parasitizing cicadas, which are attracted by singing males (Soper et al., 1976; Schniederkötter and Lakes-Harlan, 2004) and for ormiine tachinids, in which the female finds Gryllidae or Gryllotalpidae hosts by being attracted by their mating calls (Cade, 1975; Fowler, 1987; Allen et al., 1999). Lake-Harlan et al. (1999) suggested that the hearing organs of *Emblemasoma* sp. and of ormiine tachinids represent the first case of convergent evolution of homologous insect "ears."

Ormia ochracea (Bigot) has been utilized as a model organism in sound localization experiments because of its "ears," which are complex structures inside the fly's prothorax near the bases of its front legs (Robert et al., 1996). To overcome the small distance between the two "ears," the tympanic membranes of the opposite ears are directly connected mechanically, allowing resolution of nanosecond time differences. In a bionomic approach to this "finely tuned" strategy, different research groups have designed new types of specific microphones inspired by *O. ochracea*'s directionally sensitive hearing system (Miles et al., 2009; Zhang et al., 2018). Knowledge of the host searching behavior and its final acceptance could be important for the rearing of this tachinid. For this purpose, it could be also considered that *O. ochracea*, which may larviposit on or near the hosts, deposits part of its larvae around a singing cricket. Besides the vocalist, these larvae (of the planidium type) may also locate and parasitize other crickets attracted by the call, including the nonsinging females (Walker and Wineriter, 1991).

In the case of indirect oviposition or larviposition strategy, adult females use cues (physical and chemical) for locating only the host habitat or microhabitat. For instance, a substance isolated from *H. virescens* larvae was found to elicit larviposition in the host environment by *A. marmoratus* (Nettles and Burks, 1975). Physical factors associated with the host food plant (such as shape, size, and leaf color) were found to be essential for successful host microhabitat location and oviposition by the tachinid *Pseudogonia rufifrons* (Wiedemann), which oviposits microtype eggs on the leaves of certain plants (Mellini et al., 1980). In the tribe Goniini, no stimuli from parasitoid eggs attract the host larvae. The contacts between the host and parasitoid eggs laid on host food occur passively and accidentally, and the eggs hatch only when ingested by the host. Higher mortality of eggs or first instars occurs in indirect oviposition strategies than in direct ones, and thus the former is associated with higher parasitoid fecundity (several thousand eggs). When host location and acceptance are directed to first instars, the specific cues involved in the detection of a suitable host are largely unknown.

Associative learning, defined as the establishment through the experience of an association between two cues or between a cue and a behavioral response (Vet et al., 1990), has been well documented for hymenopteran parasitoids. For example, *Microplitis croceipes* (Cresson) females, experienced by contact with host feces, learned to recognize and fly to different volatile odors, even novel ones like vanilla, that were associated with the feces (Lewis and Tumlison, 1988). This learning also occurs in dipterans, and so far, it has been observed in two tachinid species, *Drino bohemica* Mesnil (Monteith, 1963) and *Ormia ochracea* (Bigot) (Paur and Gray, 2011). Similarly, the capacity to avoid superparasitism by recognizing previously parasitized hosts has been documented in two solitary tachinids, *M. doryphorae* and *Myiopharus aberrans* (Townsend), but the mechanisms involved are presently unclear (Lopez et al., 1995). Many tachinids are optionally gregarious in part depending on the host size. However, even in gregarious species, excessive superparasitism should be avoided in favor of optimizing production, as it may result in lower size and even the death of all individuals (Mellini and Campadelli, 1997; Baronio et al., 2002). The complete development of two parasitoids (of small size) in one host has also been rarely observed in solitary tachinids, for example, *Lydella thompsoni* Herting, an ovoviviparous species which displays indirect parasitization mode and lays eggs next to the holes dug by *Ostrinia nubilalis* (Hübner) (Lepidoptera Crambidae) larvae in maize stalks (Maini, 1974). All the biological aspects described here

deserve more consideration and more research as they may be important to improve the rearing procedures of dipteran parasitoids as well as the quality and performances of the flies obtained in captivity.

4.3.3 Host–parasitoid interactions

Unlike hymenopterans, dipteran parasitoids cannot suppress the host immune system or affect host physiology through secretions injected by ovipositing females (dipteran parasitoids lack a "true" piercing ovipositor) or derived from teratocytes. Yet, dipteran parasitoids display good strategies, which are especially known for tachinids, to avoid host encapsulation. Many tachinid larvae escape the host immune response and turn the host response to their advantage by forming respiratory funnels, which are sclerotized folders around the hind part of their body. Funnels may be primary, built in the host integument by first instars as soon as they enter, or secondary, formed by late-first or early-second instars in the host integument or tracheae. The tachinids that form primary funnels (*E. larvarum*) breathe atmospheric air from the beginning of their development, which permits them to grow rapidly, while those forming secondary funnels (*E. bryani* and *P. rufifrons*) breathe through their integument, and thus grow slowly, until funnel formation. The early larval stages of the tachinids that do not form primary funnels may escape the host immune response by moving into specific host tissue, such as muscle (*P. rufifrons*), or between the peritrophic membrane and gut wall (*C. concinnata*) (Baronio and Campadelli, 1979; Ichiki and Shima, 2003). A unique immune evasion structure, consisting of a "cloak" surrounding the parasitoid larval body, was described for *Drino inconspicuoides* (Baranov) an ovolarviparous parasitoid of the noctuid *Mythimna separata* Walker. The "cloak", containing both hemocytes and fat body cells from the host, was found to prevent the encapsulation of the parasitoid larvae in the host hemocel (Yamashita et al., 2019).

Another interesting aspect is host development following parasitoid attack. Parasitoids may be classified as "koinobionts" or "idiobionts"; koinobionts allow their host to continue to feed and grow beyond parasitization, whereas idiobionts permanently paralyze or kill the host before the parasitoid egg hatches (Haeselbarth, 1979; Askew and Shaw, 1986). However, dipteran parasitoids do not fit well into this classification. Many species show characteristics of both strategies, for example, tachinids (Dindo, 2011) and bombyliids (Yeates and Greathead, 1997). In Tachinidae, many species [*P. rufifrons*, *Pseudoperichaeta nigrolineata* (Walker), and *A. marmoratus*] exhibit a high degree of physiological integration with their host. The development of these species is dependent on host hormones and their first instar larvae require the host larva to reach maturity or the pupal stage before molting to the second instar (Baronio and Sehnal, 1980; Grenier, 1988b). As a consequence, the duration of their larval development is widely influenced by host age at parasitization (Mellini, 1986). The life cycle of *P. nigrolineata* is synchronized with one of its hosts *Ostrinia nubilalis* (Hübner). There are four phases of the parasitoid development requiring a hormonal signal from the host: (1) the start of the growth of the newly hatched larvae at the host ecdysis from instar III to IV, (2) the first molting at the host ecdysis from instar IV to V, (3) the growth resumption in the second instar at the ecdysteroid rise in the middle of instar V or at diapause break, and (4) the second molting at host larval–pupal apolysis. Thus, for this tachinid, the presence of 20-OH ecdysone in artificial media is necessary to trigger the first molt (Grenier, 1988b; Ramadhane et al., 1987, 1988). Other tachinid and sarcophagid parasitoids do not show developmental synchrony with their host and develop continuously until pupation. These species, especially those that form primary integumental respiratory funnels (*E. larvarum*), grow quickly following the attack and kill the host rapidly, thereby behaving as zoonecrophages for most of their larval life. The degree of complexity of host–parasitoid interaction and the extent of host development following the attack are related not only to developmental synchrony but also to respiration strategies. In fact, the tachinids that do not depend on host hormones, but form secondary funnels, grow continuously until pupation and keep the host alive longer than those building primary funnels. For different tachinid species, for example, *E. bryani*, *C. concinnata*, *Pales pavida* (Meigen) and *T. pennipes*, host–parasitoid interactions have also been found to be widely influenced by host age at parasitization (Coulibaly and Fanti, 1992; Caron et al., 2010; Nakamura, 2018; Francati et al., 2019).

Belshaw (1994) suggested that, similar to hymenopteran parasitoids, tachinids exhibiting a complex life history are generally less polyphagous than those that display rapid development. However, there are tachinids with a relatively narrow host range despite their independence from host hormones (*P. sylvestris*), whereas a high level of polyphagy is shown by several species displaying complicated host–parasitoid interactions (*C. concinnata*) (Godwin and Odell, 1984). In tachinids, polyphagy seems to be connected to the parasitoid's ability to avoid the host encapsulation response and/or indirect oviposition strategies (Askew and Shaw, 1986). In vivo production of species with a complex life history (whether based on respiration mode or hormonal interactions with the host) is not necessarily complicated, even on factitious hosts. For instance, the larval–pupal parasitoid *P. rufifrons* has successfully been cultured for many years in laboratory conditions on its factitious host *Galleria mellonella* (L.) (Mellini and Coulibaly, 1991). In contrast, host–parasitoid relationships may deeply influence the success of in vitro culture because tachinids exhibiting

developmental synchrony with the host are known to be difficult to rear on artificial media (Thompson, 1999). Independent of hormonal interactions, respiration adaptations of the parasitoid larvae have a considerable impact on methods of in vitro rearing. Species that build primary funnels need to stay in contact with air from the first instar, which makes liquid media unsuitable for their culture. In vitro development may be more problematic for parasitoids that induce the formation of secondary, rather than primary, funnels (Dindo, 2011).

Aspects related to host−parasitoid interactions are known for only a few cryptochaetid species, such as *C. iceryae* (gregarious) and *C. grandicorne* Rondani (solitary). The larvae of these endoparasitoids do not build respiratory funnels but breathe through two caudal filaments containing tracheae, which become entangled with the host tracheae. Pupation may occur inside or outside the host scale carcass (Thorpe, 1931, 1934).

The host−parasitoid interactions displayed by *Pseudacteon* phorids with their fire ant hosts are rather complex and peculiar. The fly larvae develop and build respiratory structures in the head capsule of the host ant, which displays altered behavior and is finally decapitated. Larvae turn into pupae within the detached head capsule (Porter, 1998; Mathis and Philpott, 2012). Due to this manner of pupation and difficulties involved in the collection of fertilized eggs, in vitro rearing of *Pseudacteon* would be difficult to attain on a large scale (Vogt et al., 2003). Conversely, sarcophagids attacking advanced larval stages or pupae of lepidopterans, such as *A. housei* and *Agria affinis* (Fallen), behave as zoosaprophages for most of their development and therefore show simple host− parasitoid interactions. Because of this characteristic, *A. housei* was successfully reared in vitro for many generations on different types of media (House and Traer, 1949).

4.4 Production techniques

4.4.1 In vivo production

Successful in vivo production depends on different factors, which are briefly illustrated in this section.

4.4.1.1 Larval food: natural hosts versus alternative hosts

The classical method for the production of entomophagous insects is the natural tri-trophic system, which includes the plant (or other natural food), host insect, and parasitoid. By simplifying the system, we could make parasitoid production easier.

The first step consists of replacing the plant, or other natural food, with artificial diets to grow the insect hosts. There are many examples, especially with lepidopterous larvae used as hosts for parasitoid rearing (King and Leppla, 1984; Cohen, 2015). For instance, in Brazil, *L. minense* and *P. claripalpis* were reared on their natural host *Diatraea* spp., which was reared on an artificial diet to avoid the use of sugarcane (Rossi and Fowler, 2003). Systems based on rearing the natural host appear suitable for some tachinid species, including *C. concinnata*, *B. pratensis*, and other parasitoids of *L. dispar* (Odell and Godwin, 1979; Bourchier, 1991). Also, *T. pennipes*, *L. franki* and *Nemorilla maculosa* Meigen, were reared on their natural hosts, respectively, *N. viridula* (Giangiuliani and Farinelli, 1995), the weevil *Metamasius quadrilineatus* (Champion) (Suazo et al., 2006), and the cowpea lepidopterous pest *Maruca vitrata* (Fabricius) (Agbessenou et al., 2018). The natural hosts of all these tachinids could be cultured without difficulties on either plants or artificial diets.

Natural hosts were also used to multiply nontachinid dipteran parasitoids, including the cryptochaetid *C. iceryae*, which developed on *I. purchasi* growing on 17 plant species (Mendel and Blumberg, 1991), and the sarcophagid *A. affinis*, which was reared for many generations on *C. fumiferana* (House, 1967). Conversely, attempts of rearing the bombyliid fly *Exhyalanthrax* spp., which are parasitoids of the tsetse fly, on a natural host were unsuccessful (Markham, 1986). Vogt et al. (2003) described the mass production of the phorid *Pseudacteon curvatus* Borgmeier on its natural host, the red imported fire ant *Solenopsis invicta* Buren, which was fed a standard diet of live crickets, occasionally supplemented with other components. Another phorid, *Pseudacteon bifidus* Brown and Morrison was reared on field-collected minor workers (the preferred caste) of its natural host *S. geminata* (Porter and Plowes, 2018).

The effect of host food, whether natural or artificial, should not be overlooked as it may influence parasitoid survival and quality (Thompson and Hagen, 1999). For example, Reitz and Trumble (1997) observed increased mortality of *A. marmoratus* when it was cultured on the natural host *Spodoptera exigua* (Hübner) fed three furanocoumarins derived from *Apium* sp. The effects of these allelochemicals on the tachinid were found to be mediated through their effects on the host larva. Host-plant quality was found to influence the size of the tachinid fly *Austrophorocera* sp. through its effect on the size and performance of the host *Euclea delphinii* Boisduval (Lepidoptera Limacodidae). The plants that resulted in the highest lepidopterous cocoon mass and survival (i.e., black cherry, white oak, and pignut hickory) also yielded the largest parasitoid flies (Stoepler et al., 2011).

Alternative hosts such as the lepidopterous larvae of *G. mellonella* are often easier to rear than natural hosts. The first studies with a parasitoid species should however start with rearing conditions similar to those observed in nature in order to obtain information on its basic biological, physiological, and behavioral characteristics. *Phryxe caudata* was first reared on its natural host *T. pityocampa* (Biliotti, 1956) and later on the factitious host *G. mellonella* (Delobel and Laviolette, 1969). The use of this factitious host was much more convenient and safer for laboratory rearing, especially taking into consideration the problems related to manipulation of the natural host, which bears dangerous urticating hairs. Many physiological parameters were obtained through laboratory research thanks to the rearing of this tachinid on *G. mellonella* (Bonnot et al., 1976, 1984; Grenier et al., 1986). Similarly, *P. nigrolineata* [= *Pseudoperichaeta insidiosa* (Robineau-Desvoidy)], which parasitizes *O. nubilalis* in nature, was reared in the laboratory on *G. mellonella* (Grenier and Delobel, 1982); at least 21 other tachinid species were also reared on *G. mellonella* (18 are listed in Grenier, 1986). In Cuba, *L. diatraeae* was multiplied first on *Diatraea* spp. larvae, but later mass production occurred on *G. mellonella*. Indigenous to Cuba, *L. diatraeae* has been multiplied and released in sugarcane fields since 1945. An artificial diet for *G. mellonella* larvae was developed to improve the production system without reducing the parasitoid quality (Alemán et al., 1999). *Galleria mellonella* supplies several of the nutritional requirements for the development of many tachinid and hymenopterous parasitoids (Campadelli, 1988) and also for entomopathogenic nematodes (Ehlers and Shapiro-Ilan, 2005). Water-free artificial diets are a possibility for rearing *G. mellonella*. The absence of water results in reduced mold contamination, allowing the elimination of fungicides from the diet (Mellini and Coulibaly, 1991).

4.4.1.2 Infestation mode

Extensive research concerning the rearing technique of *P. rufifrons* on *G. mellonella* revealed that the parasitization mode is crucial (Mellini and Coulibaly, 1991). In nature, *P. rufifrons* oviposits microtype eggs on foliage, and natural hosts (noctuid lepidopterans) become infested by eating "contaminated" leaves. In captivity, oviposition was obtained on wax substrates simulating leaves, and the parasitoid developed in *G. mellonella* larvae after they had fed on these artificial "leaves." The highest percentages of parasitism were obtained when eight eggs per larva were ingested.

Another tachinid displaying an indirect oviposition strategy (via planidia), *A. marmoratus*, was successfully reared on both natural hosts and *G. mellonella*. Parasitization was either performed by exposing host larvae to maggots larviposited on pieces of pleated filter paper (Coulibaly et al., 1993) or by using maggots that were mechanically extracted from fecund females. Female flies were placed in a 0.7% formalin−water solution and homogenized three times for 3 seconds each at 8500 rpm. Following this treatment, the uterus was ruptured and the maggots were released in the solution, which was then poured through a screen to exclude fly particles. Finally, the maggots were placed in a 1:3 hydroxyethyl-cellulose and water suspension and applied to corrugated cardboard disks with an aerosol sprayer. The disks were then placed close to the host larvae (Gross and Johnson, 1985; Gross, 1994). For *L. diatraeae*, the infestation was done on *G. mellonella* larvae maintained in an appropriate-sized glass tube plugged with cotton on its extremities. This technique allowed control of the number of planidia introduced into the tube so that the effect of planidia numbers per host on the development of the parasitoid larvae could be determined (Grenier, 1981).

Galleria mellonella can be also used as a factitious host for tachinids displaying direct oviposition strategies, including *E. bryani* (Coulibaly and Fanti, 1992) and *E. larvarum*. *E. larvarum* has been maintained in culture in the laboratory of entomology of the University of Bologna since 1992 using methods described by Dindo et al. (1999). For both tachinids, parasitization occurs by exposing host larvae to flies for 30−60 minutes (three host larvae per female in the case of *E. larvarum*). The number of eggs per host proved to be very important for the success of parasitization. For *E. larvarum* and *E. japonica*, the best results were obtained when three to six eggs were deposited on last instar hosts (Mellini and Campadelli, 1997; Nakamura, 1994). Parasitization of *G. mellonella* by *P. caudata* was done either manually by microinjection in the host of larvae obtained by dissecting the uterus of gravid females (Grenier et al., 1974) or by direct oviposition of the females onto host larvae maintained between two latticed frames (Grenier, 1977).

About phorid parasitoids, infestation occurs by exposing ant workers to flies. For both *P. curvatus* and *P. bifidus*, before exposure to fly attack, the ant workers were allowed to stay in contact with brood for 30 minutes. This way, it was possible to avoid, the "freezing" behavior (e.g., immobilized and defensive posture) often shown by fire ants when exposed to phorid attack (Vogt et al., 2003; Porter and Plowes, 2018).

4.4.1.3 Influence of host age at parasitization

Host age, another key factor for successful parasitization by tachinids (Mellini, 1986), had a significant effect on the development of *P. rufifrons* in *G. mellonella*. The best parasitoid yields were obtained by infesting the last instars (Mellini and Coulibaly, 1991). In the host−parasitoid system *H. zea—A. marmoratus*, early last instars were the most

suitable hosts for parasitism, as lower percentages of late last instars were successfully parasitized (Bratti et al., 1992). The growth parameters (development and size) of the different instars of *P. caudata* varied greatly when parasitism occurred at the lowest weight vs the maximum weight of the last instar of *G. mellonella* (Grenier et al., 1974). The tachinid *P. nigrolineata* was able to parasitize any instar of its natural host *O. nubilalis* except the first one, which is due to an efficient synchronization with its host cycle (Ramadhane et al., 1987, 1988). Advanced host larval stages are the most suitable for *E. larvarum* development, but the complete life cycle of this parasitoid was also obtained in younger larvae, unless they were too small (Baronio et al., 2002).

4.4.1.4 Abiotic conditions for preimaginal development

Temperature, relative humidity (RH), and photoperiod may all affect the success and duration of preimaginal development in tachinid parasitoids. For some of them, like *C. concinnata* in *L. dispar*, the development from the first instar to adult occurred within a relatively wide range of temperature (15.6°C−29.4°C) and humidity (20%−60%) (Fusco et al., 1978). However, most species were reared at more restricted temperature and humidity levels, for instance 27°C ± 1°C and 65 ± 5% RH for *A. marmoratus* (Gross, 1994), or 26°C ± 1°C and 75 ± 5% RH for *E. larvarum* (Dindo et al., 1999). Some species, including *P. caudata* (in *G. mellonella*) and *L. franki* (in *M. quadrilineatus*), were successfully cultured at lower temperatures (around 21°C) (Delobel and Laviolette, 1969; Suazo et al., 2006). High temperatures (over 30°C) may be detrimental to tachinid parasitoid development. For example, in the system *G. mellonella*−*P. rufifrons*, no parasitoid pupae were obtained at 35°C (Mellini and Coulibaly, 1991). A photoperiod of 16:8 (L:D) (Fusco et al., 1978; Mason et al., 1991; Mellini and Coulibaly, 1991; Giangiuliani and Farinelli, 1995; Dindo et al., 1999) or 14:10 (L:D) (Coulibaly and Fanti, 1992; Gross, 1994) was adopted for the rearing of the preimaginal stages of many tachinids. Long-day photoperiods could be necessary to prevent the host from entering diapause and inducing a developmental arrest of the parasitoid larva (e.g., *P. nigrolineata* in *O. nubilalis*) (Ramadhane et al., 1988). The tachinid *I. aldrichi* exhibits an obligatory diapause that requires a period of 4 months at a temperature near 4°C to be broken (Simões and Grenier, 1999).

A rearing system for the phorid fly *P. curvatus* was described by Vogt et al. (2003); parasitized host ants were held for 13 days at 27°C and 60% humidity, and then moved to well-ventilated boxes ("attack boxes") placed at 28°C, 80% RH, and a 12:12 (L:D) photoperiod. Emergence occurred about 10 days later. High RH was necessary for parasitization and fly survival, thus a steam-producing system was connected to "attack boxes" in order to maintain the adequate RH inside the box (between 80% and 90%). The high humidity level was also needed for the development of different species of phorid parasitoids of leaf-cutter ants (*Atta vollenweideri* Forel and *Acromyrmex lundii* Guérin-Méneville). The best rearing conditions (in terms of percentage of pupae obtained and pupal survivorship) were found to be 24°C ± 1°C, 80% ± 10% RH and a 12:12 (L:D) photoperiod (Guillade and Folgarait, 2014).

4.4.2 In vitro production

For all entomophagous insects, the main concern for their use in biological control is the production of quality organisms at a reasonable cost. In vitro rearing may be an effective tool to produce these natural enemies in the absence of hosts or prey. The availability of an artificial medium could be economically desirable by simplifying the production line. However, for dipteran parasitoids, it is more appropriate to speak of in vitro "rearing" on a relatively small scale instead of actual "production," as high adult yields have so far been obtained in only a few species on artificial media. Data on this topic are available for only Tachinidae and Sarcophagidae. The first step to succeeding in the in vitro rearing is the evaluation of the nutritional requirements of the insect to be reared. Nevertheless, there are other needs and rearing conditions to satisfy, as it is briefly described in this section.

4.4.2.1 Nutritional requirements and other needs of dipteran parasitoids

Various analytical approaches were employed to determine the nutritional requirements and define the medium composition: larval food analyses, whole-carcass analyses of the parasitoid, a nutritional balance sheet, or dietary supplementation and deletion studies, the latter being difficult to apply to endoparasitoids, due to the need to maintain stable pH and osmotic pressure. The basic qualitative requirements for dipteran parasitoids are not very different from those of free-living insects, but some differences exist, particularly concerning aromatic amino acids and surfactants.

4.4.2.1.1 Nitrogen sources

Nitrogen sources could be provided as proteins, peptides, or free amino acids. Dipteran parasitoids are carnivorous insects that exhibit very fast growth during larval stages (Grenier, 1980), especially when they have access to atmospheric oxygen through respiratory funnels. Thus, they need a protein-rich medium with a well-balanced composition to avoid the loss of time and energy in conversion metabolism. The 10 usual essential amino acids are required, but other ones are highly beneficial for normal growth. In Tachinidae, aromatic amino acids are required, especially at the end of larval development for cuticle tanning (Bonnot et al., 1976). Some tyrosine-rich dipeptides, such as β-alanyl-tyrosine, are present in mature tachinid larvae, for example in *P. caudata* (Delobel and Bonnot, 1976). To maintain acceptable levels of osmotic pressure, part of the essential amino acids must be provided in proteins or peptides, but some free amino acids may be required in some species. Casein, lactalbumine, ovalbumine, serumalbumine, soybean extract, and yeast are commonly used as crude proteins or hydrolysates. Supplying aromatic amino acids is complicated because some free amino acids may be toxic at high concentrations, like phenylalanine, or have low solubility, like tyrosine (Grenier et al., 1994; Grenier, 2012). The best way to deliver aromatic amino acids is within peptides or proteins rich in tyrosine and/or phenylalanine. However, such types of polypeptides are not common in commercially available food sources and therefore are difficult to obtain in the marketplace.

4.4.2.1.2 Lipids

Lipids are mainly considered as a source of energy. The similarity of lipid composition between the parasitoid and host in some species suggests that host lipids may be absorbed with few modifications, especially neutral lipids (Delobel and Pageaux, 1981). The addition of lipids in diets improves the survival and fecundity of dipteran parasitoids. Some polyunsaturated fatty acids are necessary for development and reproduction. Phospholipids are important as key components in cell membranes and also play a role as molecular signals. Dietary sterols are required by all insects (House, 1961). Lipids may be supplied as free fatty acids or triglycerides, but their incorporation within aqueous base formulations requires surfactants. Free fatty acids are toxic for some species, but the degree of toxicity may depend on the emulsifying agent used (Thompson, 1977). Moreover, surfactants could have detrimental effects on larval respiration by modifying the cuticle characteristics near spiracles (Grenier et al., 1994).

4.4.2.1.3 Carbohydrates

They are usually considered as a source of energy, and hence a specific qualitative supply is not required. Trehalose, the most common nonreducing disaccharide in insects, plays an important role in the general metabolism (Thompson, 2003). To maintain the osmotic potential at an acceptable level, oligosaccharides (e.g., glucose) could be replaced by polysaccharides (e.g., glycogen). Sucrose may act as a feeding stimulant (Cohen, 2015).

4.4.2.1.4 Miscellaneous

Other nutrients, such as vitamins, organic acids, nucleic acids, and minerals, are required in addition to the three basic types of nutrients (proteins, lipids, and carbohydrates) (Grenier et al., 1994; Thompson, 1999). These requirements are generally similar among insects, but they are poorly investigated in dipteran parasitoids. Nevertheless, inorganic cations, especially a proper ratio between Na and K, were found to be critical for some species (House, 1977).

4.4.2.1.5 Other needs

Important physiological conditions need to be fulfilled besides nutrition to ensure complete in vitro development of dipteran parasitoids, including physicochemical factors, respiration, excretion, and hormonal titers. The pH must be stabilized between 6.5 and 7.5, even if some species seem highly tolerant to extreme levels, such as *E. bryani* (Nettles, 1986). In different tachinid species, the optimum dietary osmolarity varies between 350 and 450 mOsm (Grenier, 2012).

The presentation of the food as a liquid medium, gelled or supported by absorbent cotton, is crucial for the fulfillment of larval respiration. The open functional gut in many tachinid larvae is an important factor to be taken into consideration. It becomes necessary to supply diet medium in large quantities or regularly renew it to reduce its degradation by excretory products. The life cycle of many Tachinidae is closely dependent upon the host hormonal levels, but this dependence may not be so strict in an in vitro system. The life cycle of the tachinid *P. caudata* is synchronized with its host cycle in nature, but it does not need hormones during the first and second molts when reared in vitro (Grenier et al., 1975). Nevertheless, the addition of 20-OH ecdysone to the media was necessary to trigger the first molt in *P. nigrolineata* and *P. rufifrons* (Grenier, 1988b; Fanti, 1991). Other tachinids, such as *L. diatraeae*,

E. bryani and *E. larvarum*, which develop independently of host hormones, can also develop from egg to adult in media devoid of hormones (see Section 4.3.3) (Grenier et al., 1978; Nettles et al., 1980; Mellini and Campadelli, 1996).

4.4.2.2 In vitro rearing

Early successful attempts to rear dipteran parasitoids on artificial media were obtained in sarcophagid flies attacking advanced host larval stages and involving simple host−parasitoid relationships. Research mostly focused on *A. housei*, which was reared from egg to adult with high adult yields in media based on raw materials (salmon and fresh pork liver) (House and Traer, 1949; Coppel et al., 1959) and on chemically defined media containing amino acids, RNA, dextrose, inorganic salts, B vitamins, choline, inositol, and agar as a gelling agent (House, 1954). Subsequently, House (1966) showed that the inclusion of vitamin E was essential for reproduction and continuous in vitro culture of this parasitoid. Other successful attempts at rearing sarcophagid flies in vitro were attained in the 1950s (Arthur and Coppel, 1953; Smith, 1958).

All subsequent research on in vitro rearing of dipteran parasitoids has focused on tachinids, which have generally proven to be more difficult to be reared on artificial media than sarcophagids because of their more complex interactions with the host and more specific nutritional needs. Limited success was obtained with species displaying developmental synchrony with the host. For instance, *P. caudata* was reared in vitro only from the first to the third larval instar (Grenier et al., 1975). The medium formulation was based on the body composition of the fly larvae and was possibly deficient in protein, but supplementing it with free amino acids, beyond a certain level, was detrimental to parasitoid development due to excessive osmotic pressure (Bonnot et al., 1976). *Pseudogonia rufifrons* was reared from first instar larva to adult (with very low yields) on a subnatural medium based on *G. mellonella* pupal homogenate (Bratti and Monti, 1989) and on a medium containing bovine serum as the main ingredient, combined with trehalose, chicken egg yolk, and *G. mellonella* pupal hemolymph (Mellini et al., 1994). In both cases, host material (obtained from young pupae and thus rich in ecdysteroids) proved essential to promoting the parasitoid molt from the first to the second instar. Subsequently, Fanti (1991) showed that the first molt could be obtained by supplementing an artificial medium devoid of insect components with 20-OH ecdysone. *Archytas marmoratus*, another species exhibiting complex behavioral and physiological interactions with the host, was reared on a medium from Nettles et al. (1980) and on veal homogenate-based media, but parasitoid growth and development were poor despite the addition of lepidopterous pupal extracts (Bratti, 1994; Farneti et al., 1997). Limited results were also obtained with in vitro rearing efforts of *Lydella thompsoni* Herting, a larval parasitoid of *O. nubilalis*. Synchronization with the host life cycle in south France requires parasitization of a secondary host (*Archanara* spp., noctuids developing in reeds) for the first spring generation of *L. thompsoni* (Galichet et al., 1985; Plantevin and Grenier, 1990). In particular, the first instars failed to molt to the second instar on media based on pupal extract, either of *O. nubilalis* or of two nonhost lepidopterous species (Bratti, 1994). According to the author, this failure was possibly due to the fact that the extract was derived from an unsuitable host stage (the pupa) and/or nonpermissive hosts of *L. thompsoni*.

Complete in vitro development on an insect-free artificial medium has been obtained only for tachinids that do not display developmental synchrony with their hosts. For example, *L. diatraeae* was the first tachinid successfully reared in vitro from the first instar to adult (although only a few flies were obtained). The rearing was performed on an artificial medium gelled with agarose and containing organic acids, inorganic salts, amino acids, water- and fat-soluble vitamins, gelatin, protein hydrolysates, glycogen, adenosine triphosphate, lecithin, corn oil, and cholesterol (Grenier et al., 1978). Subsequently, *E. bryani* was reared from first instar larva to adult on a medium with a similar composition combined with other ingredients (including soy flour and chicken egg yolk). Yields reached as high as 46% of the parasitoid maggots, but only after the latter had been allowed to develop in the host for the first 18−24 hours before being transferred onto the artificial media. It was estimated that an average of 2000 flies/L of medium could be obtained (Nettles et al., 1980; Bratti and Nettles, 1992). Another parasitoid of lepidopterous larvae, *P. laxa*, was reared from first instar larva to adult on the same media, with low yields, whereas yields of about 80% were obtained on a subnatural substrate based on pupal homogenate of the host *H. zea* (Bratti and Nettles, 1995). Host material improved dramatically the in vitro growth and development of *P. laxa* (despite its nondependence on host hormones), possibly because it better satisfied the parasitoid nutritional needs as compared with the media devoid of insect components.

The best results rearing a tachinid parasitoid on artificial media have been obtained with *E. larvarum*. Besides displaying a nonsynchronized development with the host, this parasitoid also builds primary integumental respiratory funnels and thus has a simpler life cycle than the species mentioned in this chapter, which all form secondary funnels. These characteristics, in addition to polyphagy and gregariousness, make this parasitoid particularly suitable for in vitro rearing (Dindo, 2007). The first artificial medium for *E. larvarum* was combined with *G. mellonella* pupal homogenate

(Mellini et al., 1993), but host components were progressively deleted in subsequent studies. Different insect-free media were developed, based on skimmed milk (Mellini and Campadelli, 1996; Dindo et al., 2010), and tissue culture media (TNM-FH) (Bratti et al., 1995), or veal homogenate (Dindo et al., 1999). The media also contained different amounts of yeast extract, chicken egg yolk, and other additives (sucrose, fetal bovine serum, or wheat germ) and were gelled with agar. Physical support is very important for successful in vitro rearing of *E. larvarum* because, in living hosts, its larvae breathe atmospheric oxygen from the beginning of their development through the primary funnels, as explained in Section 4.3.3. Fecund adults were obtained on different media, with puparial yields (based on eggs) and adult emergence (based on puparia) reaching as high as 55% and 70%, respectively. These yields were comparable to those usually obtained from conventional in vivo rearing. On the tissue culture medium and a veal medium, *E. larvarum* was reared for five and three generations, respectively, with no drops in adult longevity and fecundity (Bratti et al., 1995; Dindo et al., 1999).

Moreover, Mellini and Campadelli (1999) demonstrated that *E. larvarum* can be mass produced in glass Petri dishes (easier to manage) instead of multiwell plates. Further improvements in the in vitro rearing procedure included the use of absorbent cotton in lieu of more expensive agar (Dindo et al., 2003).

4.4.2.3 Continuous in vitro culture

Continuous in vitro culture of dipteran parasitoids with complete exclusion of the host, which implies direct oviposition on the artificial substrate, has been achieved only for sarcophagid flies (e.g., *Sarcophaga aldrichi* Parker). This parasitoid was reared for many generations on pork liver and fish from larvae deposited on the medium by ovoviviparous females (Arthur and Coppel, 1953). Conversely, this goal has not been reached for any tachinid. The in vitro rearing of tachinid parasitoids is usually carried out by removing eggs or larvae from previously parasitized hosts or by dissecting gravid females in a physiological solution and placing them on the artificial media. Studies leading to eliminating the host from some steps of the parasitoid line of production have been performed on species showing indirect oviposition strategies, like *P. rufifrons* using artificial leaves made of bee wax (Mellini et al., 1980). Hatching, which normally occurs after the egg has been ingested, was induced by centrifuging the eggs at 1000 rpm for 10−15 seconds. The newly hatched larvae were viable and could be used for in vitro rearing trials (Mellini and Campadelli, 1989).

In the case of *E. larvarum*, which oviposits directly on the host body, captive parasitoids oviposit most eggs on host larvae. However, whether hosts are available or not, many eggs are also indiscriminately oviposited on the cage surfaces and are usually lost. Starting from this observation, Dindo et al. (2007) used eggs oviposited on a plastic sheet to rear *E. larvarum* on a medium based on skimmed milk developed by Mellini and Campadelli (1996). These out-of-host eggs proved competitive with those removed from *G. mellonella* larvae to rear *E. larvarum* in vitro, in terms of immature survival, adult production, and fly quality. This study showed that, at least for one generation, in vitro production of *E. larvarum* may be disengaged from dependence on a living host. The quality of in vitro-cultured tachinids (in terms of fecundity, egg viability, and adult survival) decreased over subsequent generations when plastic sheets were provided as oviposition substrates to parental or first-generation females (Marchetti et al., 2008). Moreover, in both studies cited in this section, the eggs oviposited on the plastic sheet were dramatically fewer than those oviposited on host larvae. Further research is needed on methods to stimulate oviposition by *E. larvarum* on artificial substrates and directly on artificial media. A better understanding of the chemical and physical cues involved in oviposition of this parasitoid could be valuable, as is shown for the congeneric species *E. japonica* where cylindrical shapes simulating hosts contributed to enhanced oviposition (Tanaka et al., 1999).

Table 4.2 provides examples of dipteran parasitoids reared on artificial media divided on the basis of the presence or absence of insect components, as suggested by Grenier and De Clercq (2003).

4.4.3 Adult maintenance

Factors such as food, available space, and other abiotic conditions may affect adult survival, mating, fecundity, and success of parasitoid rearing. These aspects will be outlined in this section, especially about tachinids.

4.4.3.1 Food and water supply

Although the nutritional needs of adult parasitoids are poorly understood, it is known that most of them feed on nectar or honeydew. These food sources, which are rich in carbohydrates, provide for their energetic requirements (Thompson and Hagen, 1999). According to Wäckers (2003), nectar might also contribute to egg maturation and other physiological processes despite the low content of amino acids, proteins, and lipids. In nature, some tachinids can feed on pollen

TABLE 4.2 Examples of dipteran parasitoids reared on artificial media with (W) or without (WO) insect-derived material (see text for details).

Parasitoid		Type of artificial medium	Development obtained
Family	**Species**		
Sarcophagidae	*Agria housei*	WO	First instar to fecund adult—many generations
	Sarcophaga aldrichi	WO	First instar (larviposited directly on medium) to fecund adult—many generations
Tachinidae	*Archytas marmoratus*	W	Second instar to pupa Third (=last) instar to adult
	Eucelatoria bryani	WO	First instar (extracted from host) to adult
	Exorista larvarum	W WO	Egg to fecund adult Egg to fecund adult (3–5 generations)
	Lixophaga diatraeae	WO	First instar to adult
	Lydella thompsoni	W	Second instar to pupa
	Palexorista laxa	W or WO	First instar to adult
	Phryxe caudata	WO	First instar to third (=last) instar
	Pseudogonia rufifrons	W	First instar to adult

(Campadelli, 1977). In captivity, adult tachinids are often provided with absorbent cotton soaked in honey- or sugar- water solutions and/or sugar cubes (Fusco et al., 1978; Mason et al., 1991; Mellini and Coulibaly, 1991; Coulibaly and Fanti, 1992; Kfir et al., 1989; Dindo et al., 1999, 2010; Sourakov and Mitchell, 2002). Honey solutions may also be gelled (Grenier, 1977; Quednau, 1993). Furthermore, the life span and fecundity of adult flies may be enhanced by providing raisins (Giangiuliani and Farinelli, 1995; Coombs, 1997) or mixtures of different carbohydrates and protein or yeast hydolysates (Campadelli, 1977), or pollen (Dindo et al., 2019). However, as emphasized by Wäckers (2003), little attention is usually given to the selection of the adult parasitoid food source based on the assumption that any sugar-rich substrate may be suitable. More attention should be focused on comparing different food sources with the aim to optimize the performance of adult parasitoids. However, adult feeding did not always prove to be indispensable. For example, although adults of the bombyliid *V. cana* refused to feed in captivity, females mated and oviposited in high numbers, but their survival was low (Du Merle, 1966). Otherwise, the longevity of adult phorids of the genus *Pseudacteon* was found to be enhanced when flies were continuously supplied with sucrose solution (Ajayi and Fadamiro, 2016).

Feeding on host hemolymph often occurs among hymenopteran parasitoids by stinging the host body with their piercing ovipositor (Jervis and Kidd, 1986), but host feeding is far less common in dipterans (Thompson and Hagen, 1999). This behavior was documented in *E. bryani*, which may perforate the host integument with a piercing organ (see Section 4.1.2) and feed on the hemolymph coming out from the puncture. Nettles (1987) demonstrated that the fecundity of this parasitoid was prolonged by host feeding, while a solution of free amino acids or bovine serum albumin was not a good substitute. Exposing host larvae to *E. bryani* in captivity may be beneficial not only for parasitization but also to allow host feeding.

Distilled water may be supplied to dipteran parasitoid adults by spraying over the cages (Quednau, 1993) or through moistened absorbent cotton in drinking troughs (Mason et al., 1991; Dindo et al., 2010). Collazo et al. (1997) described an automatic system to supply water to mass-produced *L. diatraeae* adults, which reduced production costs and improved adult quality. Purified tap water, such as reverse osmosis water, could be a good substitute for distilled water (Morales Ramos and Rojas, 2014).

4.4.3.2 Space availability and adult management

Dipteran parasitoid adults are generally good flyers. The space available (cage volume) and adult density (number of individuals per cage) are two of the parameters to be considered for survival and mating success, and this is often a

crucial phase for life cycle completion. Mating occurred easily when adults of *L. franki* were kept in large screen cages measuring 1.5 × 1.5 × 1.2 m (with 10 males and 10 females) (Suazo et al., 2006). Larger cages measuring 3 × 4 × 2 m were required for successful mating in the bombyliid *V. cana* (Du Merle, 1966). For other species like *E. larvarum*, mating was obtained in smaller Plexiglas cages measuring 40 × 30 × 30 cm, each containing 70−80 flies with a 1:1 sex ratio (Dindo et al., 2007). Suitable small-sized (30.5 × 30.5 × 15.5 cm) cardboard cages for laboratory rearing and field release of *A. marmoratus* were described by Gross (1990b). Other parameters that are crucial for mating are sex ratio, age, and light intensity. For example, in some cases, mating was best achieved when the adult sex ratio was male biased (Odell and Godwin, 1979; Zhang et al., 2003) or when newly emerged females were kept with older males (Ho et al., 2011).

For better management of parasitoid rearing, it could be helpful to know the age of the adults in relation to their reproductive physiology. A special automatic collector was designed by Grenier and Ogier (1978) for controlling the emergence of tachinid flies, allowing an accurate determination of egg maturation dynamics (Grenier et al., 1982). Estimations of the weights of *L. diatraeae* pupae may be obtained by measurements of their length and diameter (based on preestablished mathematical equations) (Grenier and Bonnot, 1983). The approach also allows the evaluation of weight and fecundity of emerging adults because adult weight correlates with fecundity.

Oviposition or larviposition substrates (hosts for direct strategists or artificial devices for indirect strategists) must be periodically exposed to the adults inside cages, as described in Section 4.4.1. The addition of suitable soil and stones to mimic the natural environment was necessary for oviposition in *V. cana* (Du Merle, 1966).

4.4.3.3 Abiotic conditions

Adult biological parameters (mating, preoviposition period, longevity, and parasitization capacity) are also affected by abiotic factors. Adults of *B. pratensis* (Meigen) could be maintained at a relatively wide range of temperatures (15°C−26°C) and humidities (50%−90%) (Odell and Godwin, 1979); but for most dipterans, in particular Tachinidae, adults were usually maintained at a restricted range of temperature and RH (Coulibaly and Fanti, 1992; Dindo et al., 1999; Suazo et al., 2006). High RH levels (more than 80%) were required by the phorid *P. curvatus* (Vogt et al., 2003) and some tachinids, such as *Argyrophylax basifulva* (Bezzi) (Godfray, 1985). The greatest longevity of the adults of phorid parasitoids of the leaf-cutter ants *A. vollenweideri* and *A. lundii* was achieved at 80% ± 10% RH and 20°C ± 1°C (Guillade and Folgarait, 2014).

The common photoperiod adopted for adult rearing is 16:8 or 14:10 (L:D), but natural light conditions of approximately 12:12 L:D were found suitable for *L. franki* (Suazo et al., 2006). Mellini (1991) reported that tachinid adults are usually active during the daytime, for instance *P. rufifrons* oviposits mostly between the sixth and the eighth hour of the photophase (Fanti, 1984), but phonotactic ormiine tachinids are rather active during the night (Fowler, 1987). Wineriter and Walker (1990) adopted a photoperiod of 16:8 to rear *O. ochracea* (Bigot), without specifying when the parasitoid adults were active.

Mating often requires high light intensity. For example, 8000−10,000 lux was necessary for *L. thompsoni* (Galichet et al., 1985). In some cases, mating also appeared to be influenced by wind conditions. A strong breeze provided by an electric fan was necessary for 50% mating success in *A. basifulva* (Godfray, 1985). However, some species like *E. larvarum* and *L. diatraeae* easily mate in captivity without special requirements of light intensity or wind speed (Grenier et al., 1982; Dindo et al., 2007). For this reason, such species are particularly suitable for mass production.

4.4.4 Sterilization and antimicrobial agents

Artificial diets and media are rich in all the nutrients and thus exposed to contaminations by bacteria and fungi. The latter are specially damaging, because their mycelia and spores can spread-out in the entire rearing system from a single infection point. Biochemical changes produced by microbes alter the nutritional value of the diet/medium (Sikorowski, and Lawrence,1994). Different techniques could be used to overcome these troubles.

Diets and media, or their components separately, can be sterilized by filtering through a 0.22-μm or a 0.45-μm filter, to remove most viruses and microorganisms. Heat processing is also used to reduce microbial contamination (Cohen, 2015). However, these techniques are not always possible, by filtration, because of the too big size of the medium or diet particles, or by heating, which could induce coagulation and denaturation of proteins, as well as degradation of some components like vitamins (Vanderzant, 1975).

Another method is the employment of gamma irradiation, which could, however, hydrolyze chemical bonds, and thus alter the tertiary structure of proteins, mainly inducing their conformational changes, especially aggregation and

crosslinking. Gamma irradiation may also affect negatively some nutritional components, as well as physical and rheological properties of irradiated diets or media, such as viscosity or consistency, thus resulting in alteration of the food intake (Piri et al., 2011; Malik et al., 2017).

Some conventional antibiotics (gentamicin, penicillin, streptomycin) are efficient to control bacteria and nontoxic for entomophagous insects (Grenier, 1994; Mellini et al., 1996; Cônsoli and Grenier, 2010). Nevertheless, it is advised to pay special attention to insects harboring symbionts, because some antibiotics, for example, tetracycline used with *Trichogramma*, could eradicate these symbionts and greatly modify the reproductive status of the hosts (Grenier et al., 2002). Some fungicides incorporated into the food for phytophagous insects (nipagine, merthiolate, sorbic acid) are often detrimental to entomophagous insects (Grenier, 1977). Nevertheless, specific fungicides used in cell cultures, such as amphotericin and nystatin, are better tolerated than more generalist products (Grenier and Liu, 1990). Moreover, high levels of antimicrobial agents could modify the diet/medium viscosity and consistency or initiate the formation of aggregations breaking up the homogeneity of the substrate. These alterations could interfere with the nutritional value of the medium and reduce the food intake of the larvae of the hymenopteran parasitoid, *Pimpla turionellae* L. The protein content of this ichneumonid wasp could be also modified when rearing occurred on media containing antimicrobial agents. Penicillin, streptomycin, rifampicin, tetracycline hydrochloride, lincomycin hydrochloride, methyl p-hydroxybenzoate, cycloheximide and sodium benzoate significantly increased the protein content of *P. turionella* pupae, but some combinations of nystatin with methyl p-hydroxybenzoate or cycloheximide decreased this content. Potent antibacterial agents, such as novobiocin, nalidixic and oxolinic acids (bacterial DNA gyrase inhibitors) affected diversely the survival and development parameters of this parasitoid reared on chemically defined synthetic media. Regrettably, according to the antimicrobial agents implicated, their levels and their associations, the results greatly varied, rendering any generalization risky (Büyükgüzel, 2001, 2002; Büyükgüzel and Içen, 2004).

Both in hymenopteran and dipteran parasitoids, another problem may be the occurrence of resistant microorganisms, following the prolonged use of broad-spectrum antibiotics in artificial media or diets (Osimani et al., 2018). This concern has prompted research on more natural antimicrobial agents, such as essential oils or hydrolates. For example, 0.05% (w/w) hydrolate of the wild bergamot *Monarda fistulosa* L. proved to be a promising candidate to replace the conventional antibiotic (0.01% gentamicin solution) in the artificial media for *E. larvarum*, as suggested also by the microbiological analyses of the media, performed at various growth stages of the parasitoid (Dindo et al., 2021). The hydrolate concentration in the medium was based on the content of thymol, one of the compounds with the highest antimicrobial activity (Di Vito et al., 2019). Fertile *E. larvarum* adults were obtained on the hydrolate-supplemented medium but at lower yields than in the standard medium with gentamicin. The concentration thus needs to be adjusted in order to obtain efficient antimicrobial activity with a minimal negative effect on parasitoid development. Antimicrobials like chloramphenicol may be added to the water source of adult tachinids (Coulibaly and Fanti, 1992), but the use of nipagin should be avoided due its toxicity (Grenier, 1977).

4.4.5 Quality control

Quality parameters and assessment procedures of entomophagous insects have been widely discussed by van Lenteren (2003). As remarked by Grenier (2009), "quality" is not an absolute concept, and its definition depends on criteria that differ according to the production aims of each entomophagous arthropod (dipteran parasitoids in our case).

No guidelines for quality control have so far been developed for any dipteran parasitoid due to the relatively few species that have been mass-produced. Research on quality control has focused on a limited number of tachinid species. The quality of in vivo-produced *L. diatraeae* has been the subject of different research works. Pintureau and Grenier (1992) showed that modifications of biological characteristics, such as development time and puparial size, were induced by long-term laboratory rearing of this tachinid on *G. mellonella*. Subsequently, Pintureau et al. (1995) compared the genetic variability of several characters (including puparial length) between a *L. diatraeae* laboratory strain and a wild strain from Cuba. The laboratory strain showed a lower genetic variability, probably due to a genetic drift or bottleneck effect; reduced genetic variability is a concrete risk of the long-term rearing of beneficial insects that must be taken into consideration. In the Cuban strain, two lines were successfully selected for puparial size (lowest and highest length), for eight generations, suggesting that such a selection could be used to improve the efficacy of this parasitoid as a biocontrol agent. Body size was found to be correlated with fitness and fecundity in *L. diatraeae* (King et al., 1976) and in other tachinids like *E. bryani* (Reitz and Adler, 1995) and *C. concinnata* (Bourchier, 1991). In Cuba, attempts were made to maintain (and even improve) the quality of mass-reared *L. diatraeae* by modifying the composition of the *G. mellonella* diet, while taking into account production costs. A diet composed of Torula yeast, glycerin, corn, soybean flour, wheat, and sugar guaranteed the development, survival, and reproduction of both the host and the

parasitoid. The *L. diatraeae* adult emergence, egg fertility, and flying ability were comparable to those obtained with conventional host diets, which included wax and honey (both expensive ingredients). The parasitism rate of the target host *D. saccharalis* did not differ significantly between laboratory-cultured and wild-collected *L. diatraeae* under greenhouse conditions (Alemán, 2000; Vidal et al., 2002). Both, fecundity and flying ability (an important characteristic for augmentative release) were negatively affected in the laboratory population when compared to the wild population (Alemán et al., 2001).

Host weight is often correlated with the tachinid weight and indirectly influences fecundity or other parameters related to the parasitoid body size. Medium composition may also affect different characteristics of insect hosts and subsequently impact their parasitoids (Cohen, 2015). As shown for *L. diatraeae* and other tachinid species, host diet is very important for parasitoid quality. For instance, *A. marmoratus* puparial weight and other characteristics such as longevity were affected by the host diet (Gross et al., 1996). For gregarious species (*Eucelatoria* sp., *L. diatraeae*, and *E. larvarum*), puparial weight may also be affected by the number of parasitoids per host (Ziser et al., 1977; Grenier, 1981; Mellini and Campadelli, 1997).

Quality loss due to inbreeding depression (i.e., reduced fitness in a population as a result of breeding between related individuals) is a well-known risk of long-term insect rearing. Nakamura (1996) showed that, for laboratory-cultured *E. japonica*, the rates of adult emergence decreased in the F2 generation under inbreeding conditions. He suggested adopting rotational breeding by pairing females with unrelated males in small and medium-scale laboratory rearing to prevent this problem. However, as emphasized by Grenier and De Clercq (2003), in the absence of inbreeding depression, crosses between different inbred strains of the same species could be a useful tool to select favorable characteristics or minimize the harmful effects of laboratory adaptation. The final objective remains the optimization of the efficiency of a parasitoid strain for biological control applications.

Quality control is even more important for in vitro production because there is a higher probability of poor parasitoid performance compared to in vivo rearing. Some parameters to be considered for comparison of artificially vs naturally reared entomophagous insects were suggested by Grenier and De Clercq (2003). Parasitism of the natural host *L. dispar* by *E. larvarum* reared on a skimmed milk-based medium was compared in the laboratory and in semifield conditions with parasitoids reared on the factitious host *G. mellonella*. No significant difference in percentages of successfully parasitized *L. dispar* larvae was found between the in vitro and in vivo-produced *E. larvarum* in laboratory studies. The semifield trials were conducted in a cork forest in northern Sardinia, Italy, and each experimental unit consisted of a tree branch wrapped in a screen net. Five *L. dispar* mature larvae were released into the net with a couple of in vitro or in vivo-reared *E. larvarum*. The host larvae were removed after parasitoid flies died, which occurred within 48−72 hours after release. In vitro-reared females oviposited fewer eggs and induced lower host mortality than the in vivo-reared ones (Dindo et al., 2002). Subsequently, Dindo et al. (2006) compared several biological and biochemical traits among *E. larvarum* reared on a milk medium, a veal homogenate-based medium (Dindo et al., 1999), and factitious host *G. mellonella*. Surprisingly higher puparial yields and weights were obtained in both artificial media than in host larvae; however, there was no significant difference in fecundity among the treatments. In another experiment with females that emerged from puparia of similar weight, the host-reared flies produced significantly more eggs than the milk medium-reared ones. For in vitro-reared *E. larvarum*, therefore, puparial weight alone is not a reliable quality criterion to predict other traits like fecundity. Chemical analyses of *E. larvarum* mature larvae reared on both media showed lower amino acid content with deficiency of aromatic amino acids and excess of proline compared to the in vivo-reared parasitoids. The amino acid imbalance found in the in vitro-reared larvae could explain the lower number of eggs produced by the developing females, but this has not been experimentally proven (Dindo et al., 2006).

The quality of the in vitro-reared *E. larvarum*, especially in the long run, may be improved by adding host components in the artificial medium (Mellini et al., 1996). Grenier and De Clercq (2003) suggested that insect additives (whether from permissive or nonpermissive hosts) may optimize the media or diets for entomophagous insects, but economical insect-derived components should be used. Based on these considerations, the insect material-free artificial medium developed by Mellini and Campadelli (1996) for *E. larvarum* was supplemented with hemolymph derived from the black soldier fly, *Hermetia illucens* L., or the Chinese oak silkworm, *Antheraea pernyi* (Guérin-Méneville) (Dindo et al., 2016). Both hemolymphs can be obtained at reasonable costs because *H. illucens* can be easily raised on organic waste streams (Nguyen et al., 2015; Wang and Shelomi, 2017), and *A. pernyi* hemolymph is a by-product of the silk industry. On the medium enriched with *H. illucens* hemolymph the in vitro rearing of *E. larvarum* improved compared with Mellini's and Campadelli's insect material-free medium in terms of development times and percentage of eggs which produced puparia. *Antheraea pernyi* hemolymph, instead, proved to be less adequate as an ingredient of artificial media for this tachinid.

4.4.6 Storage and shipment procedures

Quality control is also important during the storage and shipment processes of entomophagous insects. Leopold (2007) emphasized the importance of preserving entomophagous insects at low temperatures to extend their shelf life, and Coudron (2007) discussed the possibility of improving their cold storage in egg, immature, as well as adult stages. Each stage responds differently to short- and long-term storage at low temperature, and their ability to tolerate cold storage depends on the nutrient origin (artificial vs. natural food). In particular, it was better to feed adults of *Podisus maculiventris* (Say) on natural food, instead of an artificial diet, for long-term storage at 10°C. Conversely, it is more advantageous to rear this bug on an artificial diet instead of natural food for short-term storage. This approach might also be used with dipteran parasitoids.

Storage of natural enemies is usually done by placing them, often at immature stages, at temperatures between 4°C and 15°C for short periods (van Lenteren and Tommasini, 2003). Data on storage of dipteran parasitoids are limited to tachinids. Fusco et al. (1978) showed that *C. concinnata* may be stored at 10°C−15.6°C as developing maggots in *L. dispar* larvae for up to 2 weeks and as puparia for 2−4 weeks. Also, *Sturmiopsis inferens* (Townsend) was stored in the pupal stage for 30 days at 15°C without any adverse effects on fly emergence, female mating, and male longevity, whereas female fertility was slightly reduced (Easwaramoorthy et al., 2000). Leopold (1998) provides examples of tachinid storage as puparia for 8 months under diapausing conditions and suggests that dormancy could be exploited in developing cold storage techniques to facilitate mass rearing. Gross and Johnson (1985) reported that *A. marmoratus* maggots extracted from females, stored for 14 and 21 days at 13°C, yielded 89% and 51% parasitization of *H. zea* larvae, respectively. Extracted maggots could not be maintained at 13°C for more than 3 days, as longer storage periods led to rapid decline of successful parasitization. Cryopreservation is a delicate technique, even for insect embryos (Leopold, 1998). It was applied to eggs of different pentatomid species [e.g., *N. viridula* and *Piezodorus guildinii* (Westwood)], which were stored for 6 months in liquid nitrogen (−196°C) and then successfully parasitized by the hymenopteran egg parasitoids *Trissolcus basalis* (Wollaston) (for *N. viridula*) and *Telenomus podisi* Ashmead (for *P. guildinii*) (Doetzer and Foerster, 2013). To the best of our knowledge, this technique has not yet been applied to any dipteran parasitoid.

Short-term storage (5 days) of in vitro-reared *E. larvarum* was possible at 15°C or 20°C. When the medium with eggs was restored to the standard rearing temperature of 26°C, the eggs hatched and the parasitoids developed to the adult stage; however, fewer adults were obtained compared to controls, especially from eggs stored at 15°C (Benelli et al., 2017). Moreover, 1-day-old *E. larvarum* puparia could be stored for 21 days at 15°C, with no significant detrimental effects on adult emergence, longevity, and female fecundity. Storage for the same period at 5°C or 10°C; however, resulted in 100% mortality (Benelli et al., 2018). Conversely, 2-day-old *E. japonica* puparia could be stored for 1−3 weeks at 4°C, despite a decrease in adult emergence, longevity and especially female fecundity (Seo et al., 2021).

Studies on shipment procedures for dipteran parasitoids are very scant. McInnes et al. (1976) described a method of shipping and handling tachinid puparia in individual gelatin capsules securely placed in holes in an expanded polystyrene block. On arrival, the blocks could be examined daily for adult emergence of tachinids and their hyperparasitoids which occurred inside the capsule to avoid the accidental release of undesirable insects.

In recent years, a number of developments have occurred in insulation, energy storage, and humidity control that provide the opportunity for improving the conditions during the transportation of insects. New vacuum panel insulation is up to 10 times more efficient than conventional expanded polystyrene, providing longer temperature control in smaller containers during shipping. Phase change materials, which absorb or release energy as they change from solid to liquid, are available with a wide range of transition temperatures that provide better options than the zero Celsius supplied by ice packs. Semipermeable membrane sachets of saturated salt solutions can maintain required humidity conditions inside sealed containers for extended periods. All these products are commercially available, allowing suitable combinations to be selected for the transport of most organisms for any desired temperature and humidity and for any required transport period while maintaining high standards of quality at reception (Pagabeleguem et al., 2017).

4.5 Perspectives and concluding remarks

The production of dipteran parasitoids is deeply linked to the utilization of these beneficial insects in applied biological control. Future research should focus on the possibility of better exploiting dipteran parasitoids in biocontrol strategies. In the past, mainly tachinids were considered for such approaches; but, more recently, some phorids have also appeared as promising potential agents for controlling ants. The examples of success achieved in biological control discussed in this chapter should stimulate efforts to increase the use of species that have proven to be effective. For instance, applied biological control of lepidopterous sugarcane borers with *L. diatraeae*, successfully performed in the Americas, could

be applied in other sugarcane-producing countries like China and other Asian countries. Moreover, the large-scale results obtained with tachinids, especially against sugarcane borers, should also stimulate the relaunch of research and application programs concerning *A. marmoratus*, *E. bryani*, and *P. laxa*, which appeared promising for applied biological control and mass production, even in vitro, in the 1980s and 1990s but were later neglected. In the literature, we have not found any information about the reasons why these experimental programs were neglected. As was evoked in the introduction, we may speculate that (1) the programs appeared to be expensive, mainly due to rearing costs—among other things, the bigger size of tachinids, compared to hymenopteran parasitoids, is unfavorable for mass production, as more food and space are necessary to multiply them; (2) the general image of flies was (and still is) unpopular, which may lead to a preference for other programs using other kinds of insects; and (3) some biological characteristics may be considered as negative for inundative biological control (e.g., adult flies are good fliers and may disperse too much from release points). The good results obtained, for instance with tachinids against sugarcane borers, may also stimulate the relaunch of programs with tachinids of similar biology and behavior, antagonists of maize lepidopterous pests. Moreover, in greenhouses, where the added value of the culture is high, some tachinids (such as *E. larvarum*) could be of interest for controlling caterpillars. Research aimed at improving the rearing technique and field release may make the use of tachinids more convenient and efficient. Future research could also be focused on the search for new dipteran parasitoid species that are suitable for biocontrol.

Increasing the use of dipteran parasitoids in applied biological control requires the improvement of their rearing techniques for mass production. Future research on these issues may deal with species that are recognized as promising biological control agents, but are not yet exploited due to difficulties encountered in their rearing. The rearing techniques of the species currently mass-produced such as *L. diatraeae* may become more efficient through the development of in vitro rearing procedures. As argued by Grenier and De Clercq (2003), even if this technology cannot completely replace the standard in vivo rearing methods, it may contribute to making the production process easier and more flexible.

Mass-rearing capability is one of the main factors that influence the selection of a parasitoid species for applied biological control. The potential of *E. larvarum* as a biocontrol agent could be exploited considering that this tachinid is one of the most promising species for in vivo and in vitro mass production. With regard to in vitro rearing in general, Grenier (2009) emphasized that the best research approach would be the constitution of "consortiums" among different institutions, either public or private, aimed at developing research programs and obtaining support from government or supranational commissions. A similar approach would benefit the production of dipteran parasitoids and their utilization in biological control. Aspects of dipteran parasitoid biology of relevance for rearing and issues related to in vivo and in vitro culture, including quality control, storage, and shipment procedures, may all be the subject of further research addressed with novel approaches. For example, nutrigenomics (genomics and proteomics applied to nutrition) may deliver helpful information on the way that nutrition affects the patterns of gene expression by using microarray techniques. The implicated genes could be recognized and typified for use as molecular markers for the characterization of the response (e.g., performance levels) of the insect to different nutritional sources (Coudron et al., 2006; Chang and Coudron, 2007). This approach, originally developed for heteropteran predators, might also be applied to dipteran parasitoids with the purpose of improving in vitro rearing and quality control techniques.

Besides nutritional considerations, the insect-rearing field may benefit from food technology principles as discussed by Cohen (2015), such as the extrusion process, flash sterilization, freeze-drying applications, and different approaches for nutrient processing. Also, more mechanization and automation of the production line have to be developed to reduce costs. As a general recommendation, it could be helpful to develop cooperation between biologists, physiologists, chemists, and physicists.

Finally, it should be remembered that insect-rearing technology is useful not only for large-scale field releases, but also on the small scale for entomological research concerning aspects of parasitoid biology, physiology, and behavior. The availability of efficient rearing techniques for tachinids or other dipterans may also be helpful in studies concerning pesticide effects on nontarget species (Marchetti et al., 2009, 2012). Aspects concerning dipteran parasitoid rearing are therefore important for biological control from different points of view, and all deserve to be exploited.

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Chapter 5

Production of hymenopteran parasitoids

Juan A. Morales-Ramos[1], John A. Goolsby[2], Chris Geden[3], M. Guadalupe Rojas[1], M.D. García-Cancino[4], B. Rodríguez-Vélez[5], Hugo Arredondo-Bernal[5], Matthew A. Ciomperlik[6], Gregory S. Simmons[7], Juli R. Gould[8] and Kim A. Hoelmer[9]

[1]Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States, [2]USDA-ARS, Knipling-Bushland Livestock Insects Research Laboratory, Cattle Fever Tick Research Unit, Edinburg, TX, United States, [3]USDA-ARA, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL, United States, [4]Laboratorio Regional de Reproducción de Tamarixia radiata (Regional Laboratory for reproduction of Tamarixia radiata), Centro Nacional de Referencia de Control Biológico-CNRF, Mérida, Yucatán, Mexico, [5]Centro Nacional de Referencia de Control Biológico (National Center of Biological Control Reference), Dirección General de Sanidad Vegetal (Directorate General of Plant Helth), Tecomán, Colima, Mexico, [6]USDA-APHIS-PPQ, Edinburg, TX, United States, [7]USDA-APHIS-PPQ, Salinas, CA, United States, [8]USDA-APHIS-PPQ, Otis AFB, MA, United States, [9]USDA-ARS Beneficial Insects Introduction Research Unit, Newark, DE, United States

5.1 Introduction

Biological control by augmentation has been applied for more than 100 years in various crop systems (Cock et al., 2010; Elzen and King, 1999; King et al., 1985; van Lenteren et al., 2018). It plays an important role today since it is used as a component to practice healthier and sustainable agriculture that protects the environment and optimizes the use of ecosystem services (van Lenteren, 1991). The success of biological control programs through mass rearing of beneficial organisms depends on quality production and an efficient release scheme (Glenister and Hoffmann, 1998).

Hymenopteran parasitoids have been used multiple times with great success to biologically control insect pests. Some of the most spectacular examples of biological control success using hymenopteran parasitoids have been under the classical approach of biological control, where natural enemies are moved from the pest's original geographic range to the newly invaded locations (DeBach, 1964; Van Driesche and Bellows, 1996). However, many important examples exist where success has been achieved using the augmentation approach, where either native or exotic natural enemies are released for control. All developmental stages of hemimetabolous and holometabolous insects are susceptible to parasitism by hymenopteran parasitoids, but most species usually specialize in parasitizing a particular life stage. The most documented uses of hymenopteran parasitoids in augmentation biological control involve the propagation and release of egg parasitoids, particularly trichogrammatids (Li, 1994; Elzen and King, 1999). Tens of millions of hectares are treated in over 30 countries using parasitoids of the genus *Trichogramma*, mostly against lepidopteran pests in diverse crops including grains, cotton, sugar cane, fruit trees, vegetables, forest, etc. and stored products (Li, 1994; Parra, 2010). The most commonly used species are *Trichogramma evanescens* Westwood, *T. pretiosum* Riley, and *T. dendrolimi* Matsumura (Parra, 2010), but Li (1994) reported 70 species of *Trichogramma* being used in biological control from which only 20 species were mass produced.

The most economic method of mass-producing *Trichogramma* is by using eggs from factitious host species. The most commonly used factitious hosts include species of stored grain moths like *Sitotroga cerealella* (Oliver), *Ephestia kuehniella* Zeller, and *Corcyra cephalonica* (Stainton) because these species are easier and less expensive to produce and can be grown on artificial diets (Postali Parra, 2010). In China, unfertilized eggs from silkworm moths such as *Antheraea pernyi* (Guerin-Meneville) and *Philosamia cynthia* Rebel are used as factitious hosts for *Trichogramma* (Parra, 2010). Eggs are manually extracted from unmated moths by cutting the abdomens and gently squishing them into a water bath fitted with a rotating apparatus with brushes. Clean eggs are lathered and air-dried in wooden trays lined with fabric (Nagaraja, 2013). Dry eggs are glued to paper cards using water-soluble glue and exposed to parasitoids in a chamber or in a room (Nagaraja, 2013).

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The increase in the use of trichogrammatid parasitoids for biological control of pests is in great part due to the advances in mass-rearing technology of lepidopteran host species (Parra, 2010). In addition, successes in the development and implementation of parasitoid artificial diets in China have made it possible to mass-produce trichogrammatids in vitro (Nagaraja, 2013). These two technological advances have provided viable ways to mass-produce trichogrammatids at a lower cost than other groups of hymenopteran parasitoids. There are few examples of mass production of hymenopteran parasitoids other than egg parasitoids. In this chapter, we will focus on describing examples of successful mass production of hymenopteran parasitoids that parasitize host life stages other than the egg. Particularly, we will describe the methods for producing and releasing these species of hymenopteran parasitoids as they are used as biological control agents against economically damaging pests.

5.2 Mass rearing of aphelinid parasitoids of the silverleaf whitefly

The development of efficient mass-rearing systems for *Bemisia* parasitoids was crucial for the implementation of classical and augmentative biological control programs for this exotic pest (Gould et al., 2008). Early systems relied on adapting methods for the production of *Encarsia formosa* (Gahan) a parasitoid of the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood). Production for the Nearctic parasitoid *Eretmocerus eremicus* Rose and Zolnerowich was developed using this system, but new production techniques were needed for the exotic Palearctic parasitoids that were imported during the biological control program, such as *Eretmocerus mundus* Mercet, *E. emiratus* (Zolnerowich and Rose) and *E. hayati* (Zolnerowich and Rose) which are unable to develop successfully in *Trialeurodes* species (Goolsby et al., 1998).

In this section, we describe several rearing techniques developed in support of augmentative biological control demonstration projects in California, Arizona, and Texas to establish new species of *Eretmocerus* and for greenhouse biological control evaluations in several states (Goolsby and Ciomperlik, 1999; Pickett et al., 2004; Goolsby et al., 2009). The species reared on *Bemisia tabaci* (Gennadius) biotype "B" using the systems described here include all of the *Eretmocerus* species that were collected in the worldwide effort to introduce more effective *Bemisia* parasitoids to the US (Kirk et al., 2000; Legaspi et al., 1996; Goolsby et al., 1998, 2005, 2009). These include some of the most effective Palearctic and North American species: *Eretmocerus emiratus*, *E. mundus*, *E. hayati*, and *E. eremicus* (Rose and Zolnerowich)

The initial rearing methods for production of *B. tabaci* and its imported natural enemies are described in this chapter. These methods were used at the USDA-APHIS-PPQ Mission Biological Control Laboratory (MBCL) in Mission, TX, and were smaller in scale than those described in the second part of this chapter. Rearing systems at MBCL used both hibiscus and eggplant as host plants for *B. tabaci*. The rearing operations were used to support small-scale colonization of parasitoids, colony maintenance, and quarantine evaluations (Goolsby et al., 1996, 1998) of new exotic *B. tabaci* cultures collected from the worldwide exploration effort. Parasitoids were reared in laboratory colonies from 1991 to 2000 to provide material for field cage evaluations in Arizona, California and Texas; for greenhouse biological control evaluations in Colorado, Mississippi, and New York; to provide seed material for the rearing operations in California, and for direct field releases throughout the US

A total of 58 populations and or species of parasitoids were reared at MBCL from 1991 to 1999. Initially, all cultures in quarantine were colonized on whitefly-infested hibiscus, *Hibiscus rosa-sinensis* L. Following release from quarantine, parasitoid cultures were increased in the laboratory production facility on both whitefly-infested hibiscus and/or eggplant, (*Solanum melongena* L.). Production in the laboratory facility allowed for stable year-round rearing, which could be used for inoculation of outdoor rearing cages. Outdoor cages were designed for the economical rearing of multiple species for field release. Together the use of these two systems resulted in the production of several million parasitoids from the 58 species and/or populations of natural enemies collected in the foreign exploration effort. A third high-output greenhouse-based production system was developed for the production of parasitoids for local augmentative release programs. This system was primarily used for the production of *Eretmocerus* spp. for release in Arizona and California.

5.3 Laboratory culture

5.3.1 Plant culture

All parasitoid and predator cultures were reared on whitefly-infested *Hibiscus rosa-sinensis* var. "Brilliant Red." This variety produced large glossy leaves that were ideal for oviposition by *B. tabaci*. Plants were grown in 15.2 cm (6 in.)

wide pots from cuttings or liners purchased from commercial nurseries in Florida. Mature plants were ready for whitefly oviposition approximately 2 months after potting. They were recycled twice and then discarded. More than 500 plants were held in rotation to supply adequate numbers of plants for the rearing colonies. Before using plants in the insect colonies, newly expanding young leaves were removed to force oviposition by Silverleaf whitefly only onto the large mature leaves. The large, mature leaves were easier to handle in the rearing process. Plants were also completely hand-wiped with towels to remove aphids, mites and other pests. Plants were watered weekly in the environmental growth chambers.

5.3.2 Whitefly oviposition

A population of *B. tabaci* collected from Mission, Texas was used to initiate the "mother" whitefly colony. Approximately 10,000 adult whiteflies were used to start the mother colony. To ensure the long-term viability of the colony, a new colony was restarted every two years, always replacing it with field-collected whiteflies from the local agricultural area near Mission, Texas (Hidalgo, Co.) in the Lower Rio Grande Valley. Mature hibiscus plants were placed in an environmental growth chamber set at 27°C and a 14:10L:D photoperiod. The lights were fluorescent with special ballasts, which had a flicker rate of more 750 cycles/ second. A combination of Vitalitet and Chromo-50® 40-watt bulbs produced a full spectrum of artificial light. Each chamber was illuminated from overhead with two each of the above fluorescent bulbs. Inside the chamber, plants were placed inside a $0.5 \times 0.5 \times 1$ m aluminum frame fitted with a white organza shroud to confine the emerging adult whitefly on the hibiscus plants. Approximately 100 mature clipped hibiscus leaves bearing fourth instar whiteflies were stapled to paper towels and placed in the cage for emergence and subsequent oviposition onto new plants. The plants were kept in the whitefly-infested shroud cage for 2 days at which point egg density reached approximately 50 eggs/cm^2. After oviposition, whiteflies were removed with a hand-held vacuum. The short oviposition period produced whitefly populations with a strict cohort on each plant, which were then held at 27°C for 7 days. After this period, whitefly had developed to mostly second instar nymphs suitable for parasitization. A portion of plants (1/15) were held for maturation of the whitefly to produce adults for the next oviposition cycle. Spider mite outbreaks (*Tetranychus* spp.) were avoided by quarterly inoculative releases of the phytoseiid predator mite, *Phytoseiulus persimilis* Athias-Henriot applied at a rate of 500 mites per 25 plants.

5.3.3 Parasitoid culture

Eretmocerus spp. (Hymenoptera: Aphelinidae) are solitary, internal parasites of whitefly that oviposit external to developing second to third instar whitefly nymphs (Fig. 5.1A and B), complete larval development in the whitefly nymphs, and pupate in mummified fourth instar whitefly larvae. Adult *Eretmocerus* emerge from the mummified whitefly larva through an exit hole cut in the dorsum of the host. *Eretmocerus* spp. void their meconia (fecal pellets) outside the body of the host whitefly (Fig. 5.1C). *Encarsia* spp. have the same general biology, except that males (from unfertilized eggs) develop as a hyperparasitoid of developing female *Encarsia* or *Eretmocerus* spp. In addition, *Encarsia* void their meconia as they pupate inside the body of the parasitized whitefly (Fig. 5.1D). Parasitoids were reared in environmental growth chambers operated under the same conditions as described above. Whitefly-infested plants were placed inside cages designed to prevent escape of the extremely small aphelinids. The whitefly-parasitoid cages were $0.25 \times 0.25 \times 0.5$ m with three sides made of white translucent Plexiglas with top and front sides from clear Plexiglas. A 10 cm hole on the top was covered in fine mesh organza for ventilation. The right side had a similar hole with a 20 cm organza sleeve which allowed access for watering and wasp collection. The left side had a 30 cm oblong hole with a sock large enough to allow for addition of potted, 15.2 cm (6 in.) wide hibiscus plants. The cages could hold a total of four hibiscus plants.

One hundred adult *Eretmocerus* parasitoids of mixed sex were aspirated into vials and released into each cage, which contained two to four hibiscus plants infested with mostly first and second instar whitefly. For *Encarsia* spp., plants with a larger proportion of third and fourth instar whitefly were used. For autoparasitic (autoparasitoids lay male eggs in the body of female larvae of their own species, or closely related taxa) *Encarsia*, like *Encarsia sophia* Viggiani, a third plant containing already parasitized whitefly was added to allow for the production of male progeny. Plants with parasitized whitefly were held for 14 days until the majority of the parasitoids had pupated (Fig. 5.1D). At this point, leaves were clipped, scanned under a dissecting scope for contamination of aphids or autoparasitic *Encarsia* spp. in *Eretmocerus* cultures and stapled to paper towels and hung up in emergence cages. If the whitefly nymphs on leaves from Eretmocerus colonies were contaminated with *Encarsia*, the unwanted *Encarsia* pupae were scraped off the leaf with a pair of forceps. As parasitoids emerged they were collected with an aspirator and used to inoculate the next

FIGURE 5.1 Field cage production system. (A) *Eretmocerus hayati* male anntenating emerging female in advance of mating. (B) *Eretmocerus emiratus* female ovipositing into *Bemisia tabaci* second instar nymph. (C) *Eretmocerus mundus* pupae inside exuviae of whitefly, note empty areas on either side of pupae which allows the parasitized whitefly to float as compared to unparasitized whitefly that sinks (this feature is used for separation of parasitoid pupae). (D) *Encarsia sophia* pupae inside exuviae, note the presence of dark meconia which are characteristic of this parasitoid genus. (E) *Bemisia tabaci*, Biotype B adult, eggs and exuviae on underside of eggplant leaf. (F) High density of *B. tabaci* adults on cumber leaves in the field in the Lower Rio Grande Valley of Texas (1994). (G) Vacuum collection of whitefly adults from the mother colony to use for infesting field cages. (H) Release of parasitoid adults onto infested plants in a field cage. (I) Field cage full of infested eggplants which have been inoculated with parasitoids; plants are mature and ready for harvest of leaves with parasitized whitefly. (J) Harvested leaves drying on racks for 1 day. (K) Harvested leaves inside Plexiglass emergence cages which are used to collect adult parasitoids. (L) Emergence cage with black shroud to force adult parasitoids toward light and into Petri dishes used for collection.

series of cages or removed for field releases or evaluation studies. The top right-hand corners of production and emergence cages were streaked with honey using an eyelash brush since the addition of honey significantly increased the longevity and fecundity of the parasitoids.

Fifty-eight species/populations of parasitoids were reared using these methods. At the peak of production in 1995, six insect production workers were required to manage the parasitoid and whitefly colonies. Periodic samples of each parasitoid colony were checked for purity using RAPD-PCR. If contamination was detected, a new culture was initiated from each of ten iso-female lines that matched the original parental RAPD-PCR banding pattern. To minimize contamination, each growth chamber contained no more than one *Eretmocerus* and one *Encarsia* species in combination. If congeneric species were reared in combination, morphological differences between adults and pupae of *Eretmocerus* and *Encarsia* allowed production workers to detect early stages of contamination. Since the *Eretmocerus* spp. from similar appearing species or different populations were difficult or impossible to distinguish using macro-morphological characters, only one *Eretmocerus* spp. was reared per chamber. These meticulous anticontamination efforts carried out by the production and genetics teams resulted in no populations being lost due to contamination during nine years of insect rearing.

5.4 Outdoor field cage production

5.4.1 Plant culture

All parasitoid cultures were reared on whitefly-infested eggplant, *S. melongena* var. "Ichiban" (Fig. 5.1E and F). This variety of eggplant produced large leaves that were ideal for oviposition by *B. tabaci*. Kale, *Brassica oleracea* "Acephala" was used from November to February because of its tolerance to cool, winter temperatures. Plants were grown from seed in 15.2 cm (6 in.) wide pots. Mature plants were ready for whitefly oviposition approximately 3 months after potting. More than 1000 6-inch plants were kept for the production cycle, with new plants started each week to maintain the supply of young vigorous plants.

5.4.2 Whitefly oviposition

As with the Laboratory Culture, a locally collected population of *B. tabaci* was used as the "mother" colony in the Outdoor Field Cage Production. The colony was replaced every two years with a new field-collected whitefly population. Mature plants were placed in 3.1×3.1 m $\times$ 1.8 m high field cages covered in 52×52 mesh Lumite Saran™ (Lumite Industries, Gainesville, Georgia) (Fig. 5.1G, H, I). Cages were pinned to the ground using large 25×1.23 cm ($10'' \times 1/2''$) soil auger screws. Cages were sealed along the bottom edges with weights made of sand-filled lengths of 4-inch fire hose. Plants and whitefly were reared under ambient outdoor temperatures, except during the coldest months from November to February (the mean minimum and maximum temperatures for McAllen, TX in January are 7.6°C and 20.1°C, respectively.). During the winter, cages were covered with clear greenhouse plastic and a single electric 12. 5-amp, 1300-W portable heater was placed in each cage, which increased the temperatures in the cages by about 5° C above ambient temperatures. Eight outdoor cages with 40 eggplants were needed for a continuous supply of whitefly for inoculation of parasitoid production cages. All the plants were watered daily on an automated irrigation timer. Liquid fertilizer was added to the irrigation water monthly, or as needed, as per label rates (20−20-20, Peter's®, Allentown, Pennsylvania). The whitefly production facility was 1.6 km (1 mile) distant from the parasitoid production facility to reduce the possibility of contamination from parasitoid colonies. As whitefly adults emerged, they were vacuumed off using a high-volume low speed ventilation box fan. The adults were collected in a fine mesh cotton organza sock for transfer to the parasitoid production facility or to inoculate new whitefly production cages (Fig. 5.1H). Approximately 10,000 whiteflies were added to each cage. Plants were monitored until whitefly egg density reached 50 eggs/cm^2, at which point adult whitefly were removed by vacuuming (Fig. 5.1G). The short oviposition period produced a defined whitefly cohort on the plants, which was held for 7−21 days ("d") at which point they contained mostly second instar whitefly nymphs that were suitable for parasitization. Spider mites and cotton aphid, *Aphis gossypii* outbreaks were avoided by quarterly inoculative releases of the phytoseiid predatory mite, *P. persimilis* or the aphelinid parasitoid *Aphelinus gossypii* Timberlake, respectively.

5.4.3 Parasitoid culture

Parasitoids were reared in the same cages with the same environmental parameters as those described above. One hundred and twenty 15.2 cm (6 in.) potted eggplants were placed in cages and allowed to mature to the 6-leaf stage, which

took approximately one month. The same number of kale plants was used during the winter months, but plants were held for 2 months prior to inoculation with whiteflies. Adult whiteflies were then added using the methods described above. Parasitoid cages were zippered with an additional Velcro flap to prevent escape or contamination of parasitoids. An on-site weather station provided degree-day information so that we could predict when parasitoids would be ready for harvest (von Arx et al., 1983).

Two to six thousand parasitoids of mixed sex were added to each cage (Fig. 5.1H), which contained plants infested with mostly first and second instar whiteflies for production of *Eretmocerus* spp., and third and fourth instars were used for the production of *Encarsia* spp. Plants with parasitized whiteflies were held from two weeks to two months (depending on outdoor temperatures) until the majority of the unparasitized whiteflies had emerged and the parasitoids had reached the pupal stage. At this point, the leaves were clipped, scanned for contamination by *Encarsia* spp., aphids, etc. and stapled to paper towels (Fig. 5.1J) and hung up in indoor laboratory emergence cages maintained at 25°C (Fig. 5.1K). Emergence cages ($1 \times 1.5 \times 1$ m) were covered with black shrouds, except for two round openings at the top facing the fluorescent lights (Fig. 5.1L). The round openings were designed for the placement of large 10 cm Petri dishes (bottom halves) (Fig. 5.1L). Emerging parasitoids gathered on the top half of the petri dish. As parasitoids emerged and gathered on the Petri dishes, they were removed and closed with the other piece of the dish, which was streaked with honey. The numbers of parasitoids were counted using a subsample on a fixed grid. Petri dishes were sealed with parafilm and placed in a temperature-controlled cabinet at 16°C with a 14:10L:D cycle. Dishes containing adult parasitoids were shipped interstate to cooperators, held for inoculation of cages, or used for field release. Aside from plant production, one person was able to manage the daily tasks of the entire outdoor production facility.

The outdoor cage production procedures provided a low cost, low input method for multiplying parasitoids. However, production was somewhat unpredictable during cooler winter months. If the mean temperature in the cage was below 16°C, the development of the parasitoids stopped. It was not unusual for the parasitoid generation time to take up to 2.5 months during the winter. Toward the end of the project, heated greenhouses were used instead of the outdoor cages for mass production. Although the production from heated greenhouses was more predictable, the cost for the structures was considerably higher than the outdoor cages. The outdoor cage method should be considered as an alternative method for multiplication of whitefly parasitoids during warmer months, especially if funds are limited.

5.5 Large-scale greenhouse-based system

A joint project by the California Department of Food and Agriculture (CDFA), USDA-APHIS-PPQ, the Imperial County Agricultural Commissioner's office, and private industry was launched in Imperial County, California, to mass-rear the most effective exotic whitefly-parasitoid species. The goal of this project was to increase the biological control of *B. tabaci* biotype B in spring melons by rearing and releasing several species of whitefly parasitoids. Rates of parasitism by native parasites of whitefly (*E. eremicus* and *Encarsia* spp.) are generally low in the spring melon crop in desert production areas of California and Arizona, which is where whitefly populations first start to rapidly increase, leading to high regional populations subsequently infesting cotton and alfalfa after the melon harvest. In addition, the mass-rearing initiative supported the classical biological control program with the goal of establishing new species of parasitoids. Benefits of this program included: the release of more than 60 million exotic species of whitefly parasitoids into the Imperial Valley, which helped establish several new species; the transfer of rearing technology; to provide new more effective whitefly parasitoids to the beneficial insect industry; and to improve the use of mechanized beneficial insect delivery systems in cooperation with industry.

5.5.1 Plant production

In the Imperial Valley, plants were grown all year in a greenhouse without artificial lights (Fig. 5.2B). Suitable plants were produced in 6–8 weeks. Eggplant seeds were planted into one-gallon plastic pots filled with peat-based soil (Redi-Earth™, Scotts, Ohio) mixed with coarse-grained sand (Play Sand, Quikrete™, Atlanta, Georgia) to make a mixture of ca. 60:40 of peat moss and sand. Osmocote™ slow-release fertilizer (14–14-14, 3–4-month formulation, Scotts, Marysville, Ohio) or a similar generic brand was mixed into the soil at a rate of 6.27 L/m^3 of soil. Before use, the soil mix was sterilized with an electric box sterilizer by bringing the temperature of the soil to 77°C for ½ hour. The main eggplant varieties used were Black Beauty and Whopper, though good results were also achieved with Asian varieties, such as Sennari and Ping Tung. Plants were grown in a greenhouse

FIGURE 5.2 Greenhouse production system. (A) Large eggplants infested with *B. tabaci* shrouded to contain adult parasitoids which have just been released. (B) Eggplants held in greenhouse for maturation of parasitoid pupae. (C) Funnel showing bulk unparasitized *B. tabaci* and parasitoid pupae floating in water that have just been removed from the eggplant leaves using a high-pressure flat fan sprayer. (D) Funnel showing separation of unparasitized whitefly that sink to bottom and a top layer of floating parasitoid pupae. (E) Parasitoid pupae drying on nylon mesh cloth. (F) Parasitoid pupae are being weighed to determine approximate numbers.

equipped with both heating and cooling, so that plants were grown at temperatures ranging from 22°C to 33°C throughout the year. Plants were provided additional fertilizer through the irrigation system with a soluble fertilizer (20−20-20, Peter's™, Allentown, Pennsylvania) at a dilution ratio of 16:1 of water to fertilizer. Under these conditions a plant usable for whitefly oviposition was produced within 5−8 weeks depending on the season. Plants that were ready for production were growing robustly with a height of 0.6−1.0 m and leaves as wide as 19 cm. Such plants had 25−40 large leaves that were suitable for production. Good plant health was the single most important factor for good parasitoid production.

5.5.2 Insect and disease control

Insect infestations caused problems in several ways. Eggplants infested with insect pests were weaker and less able to tolerate the high density of whitefly nymphs necessary for high parasitoid production. Infested plants grew more slowly, became deformed, and shed leaves in the parasitoid rearing before the parasitoids were mature. Pest infestations also reduced oviposition activity by both whitefly and parasitoids by making the plants less suitable for feeding and oviposition and also by simply taking up space on the leaf.

Infestations of *Bemisia* in the clean plant production greenhouse caused problems when they became established on a cohort of plants before plants were exposed to whitefly in the production system. Early whitefly infestation led to contamination by *Encarsia* spp. (especially *E. hispida*, De Santis), which had become a pest in the greenhouses after it had been in production. Whiteflies that became established too early also emerged as adults in the parasitoid exposure cages, resulting in heavy feeding and excreting honeydew, which weakened plants and interfered with parasitoid oviposition.

For all of these reasons, it was important to maintain a pest-free environment in the plant production greenhouse as much as possible. To reduce pest entry, greenhouse vents and doors were screened with 52×52 mesh (Lumite Saran™ Synthetic Industries, Gainesville, Georgia). A combination of pesticides and natural enemy releases were used, as needed, to keep plants as clean as possible. Pesticides with short or no residual activity were used so that treated plants could be placed into the parasitoid production system with little delay. Pesticides were frequently rotated to avoid problems with the buildup of resistance. Pesticides were tested for phytotoxicity to eggplant before use; in some cases, application rates had to be adjusted to levels lower than recommended by the label to avoid damage to eggplant leaves. Young seedlings were the most susceptible to damage from spraying and, whenever possible, spraying was avoided in plants younger than 2−3 weeks. Most of the pesticide applications during plant production were made before their introduction into the whitefly and parasitoid colonies to avoid applying pesticides directly to the insect cultures. Treatment of pests in the insect colonies was predominantly limited to releases of natural enemies, especially spider mite predators and aphid parasitoids; although exceptions are noted below.

The pesticide treatments included pyrethrin-rotenone (Pyrellin EC™, Webb Wright Corp., 2.5 mL/L), pyrethrin-piperonyl butoxide (Concern™ Miracle-Gro Lawn Products Inc, 2.5 mL/L), insecticidal soap (M-Pede ™, Mycogen Corp., 19.2 mL/L), Cyfluthrin (Tempo 2EC™, Bayer Corp., 0.3 mL/L), Azadirachtin, (BioNeem™, Safer, 23 mL/L). These pesticides were used alone at these rates or mixed with M-Pede insecticidal soap for control of the following insect pests: green peach aphid, *Myzus persicae* (Sulzer); cotton aphid, *Aphis gossypii* Glover; *B. tabaci* (Gennadius); long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti); and western flower thrips, *Frankliniella occidentalis* (Pergande). Although all pesticide treatments helped in the control of the listed pests, most of the applications were made for control of *B. tabaci* in the production greenhouse for clean eggplant, as the other pests were occasional problems that could be controlled by releases of commercially available natural enemies for aphids, mealybugs and thrips. Treatments for whitefly were made on average once a week from May to September. The most frequent treatments were insecticidal soap alternated with insecticidal soap mixed with a pyrethrin compound.

Aphids were controlled effectively by releases of *Aphidius colemani* Viereck at the rate of 2−5 per plant. Infestations of long-tailed mealybug were treated with releases of *Cryptolaemus montrouzieri* Mulsant, the mealybug destroyer, at the rate of 1−2 beetles per plant. Thrips infestations were treated with releases of the predatory mite, *Amblyseius cucumeris* Oudemans, at rates of 5−20 mites per plant. Thrips were also treated with releases of minute pirate bug *Orius insidiosus* (Say) at the rate of 1−5 bugs per plant. As minute pirate bugs are also whitefly predators, these were only released in the plant production greenhouse. Mite species that caused problems included two-spotted spider mite, *Tetranychus urticae* Koch, and broad mite, *Polyphagotarsonemus latus* (Banks). At the first sign of two-spotted spider mite infestation, plants were treated with mixed releases of mite predators: *P. persimilis, Mesoseiulus longipes* (Evans), and *Neoseiulus californicus* (McGregor). These were released at the rate of 10−20 mites per plant. Broad mite infestations and severe two-spotted mite infestations were treated with avermectin (Avid 0.15 EC™, Syngenta Inc., 0.6 mL/L). Other pests included ants, especially argentine ants, *Linepithema humile* (Mayr), which tended aphids and preyed on parasitoid pupae, and fungus gnats, *Bradysia* spp. Ants were treated with applications of hydramethylon ant bait (Amdro Pro, BASF) by putting 10−15 g in a small Petri dish placed in the corner of each whitefly or parasitoid cage. Fungus gnats were treated with soil drenches of *Bacillus thuringiensis* var. *israelensis* (Gnatrol™, Valent, 10.0 mL/L) once a week.

Occasionally the whitefly colony would become infested with undesirable parasitoids, (especially *Encarsia* spp.), greenhouse whitefly, or spider mites. Initially, badly infested colonies were destroyed, and new ones were started, but this method was a setback for production as the colonies could actively produce whitefly for 4−8 weeks. Taking advantage of the high levels of acephate resistance (nearly 100%) occurring in Imperial Valley populations of *B. tabaci*

(Steve Castle, USDA-ARS, Phoenix, AZ, personal communication), while aphelinid parasitoids, greenhouse whitefly, and spider mites were completely susceptible, infested whitefly colonies were treated with acephate (Orthene 75 S, Valent, 0.77 mL/L) to eliminate everything except whitefly populations.

5.5.3 Whitefly colony

To start a new colony, whitefly adults were collected from the field and transferred to colony cages with eggplant as described above. Initial whitefly colonies were collected in the late winter from cole crops, such as cabbage, broccoli and cauliflower where large populations of whitefly infestations could be found. Whitefly adults were collected with a gas-powered vacuum (D-VAC Vacuum Insect Net Model 24, Rincon-Vitova Insectaries, Inc., Ventura, California) fitted with a nylon organdy bag over the end of a collection hose. Whitefly adults were collected for 5 minutes periods into the bags, which were then tied off with a piece of twine and transferred to an ice chest maintained at 13°C−15°C for transport to the laboratory.

Whiteflies were released inside a $0.9 \times 0.5 \times 0.4$ m high wooden cage with a slanted glass top. A fluorescent light source was placed over the cage to attract the whitefly to the top of the glass and aid in their separation from other insects present in the collected samples. Whitefly was collected with an aspirator constructed with 0.64 cm (¼ in.) plastic tubing attached to 0.64 cm (¼ in.) aluminum tubing fitted into a 9-dram vial with a rubber plug. The aspirator was connected to an electric vacuum pump (Model G582DX-S55NXMLD-6711, 1/3 HP, 5.5 A 115 V, Gast, Mfg. Corp, Benton Harbor, Michigan) with plastic tubing of the same diameter. Whitefly adults were collected into the vials, inspected for other species, and stored at 13°C−15°C. When other species were prevalent in the collection, repeated re-transference of collected whiteflies to clean cages was necessary to obtain a pure colony. Whiteflies were transferred to the greenhouse in a 13°C−15°C ice chest to release on eggplant in cages to start a new whitefly colony.

Plants, 4−6 weeks old, were washed with a fine spray of water to remove dust and pesticide residues, damaged and yellow leaves were removed, and plants were placed into $1.8 \times 1.8 \times 1.8$ m whitefly oviposition cages inside a 6×12 m greenhouse (Fig. 5.2A). The greenhouse temperature was maintained within a range of 20°C−38°C throughout the year. Production in the late winter/early spring for the first releases on spring melons was an important time of the year for rearing. Observations indicated that when temperatures fell below 20°C, production levels of both whitefly and parasitoids were poor. Conversely, temperatures above 38°C, resulted in production decline since eggplant appeared to be less tolerant to high levels of whitefly feeding at higher temperatures and many leaves became yellow and died before parasitoids could mature.

Cages were filled with 14−20 plants per cage depending on the size of the plants. The cages were made of Lumite Saran™ (52×52 mesh) material with doors secured by zippers with a flap of fabric secured by Velcro™ to keep small insects from entering around the zipper teeth (Fig. 5.2A). Plants were watered 2−3 times per week using a drip irrigation system, depending on temperature and growing conditions. Plants were fertilized weekly with a soluble fertilizer (Peters 20−20-20) at a dilution ratio of 16:1 water to fertilizer.

When a target density of 15 or more whitefly/cm^2 was reached in the oviposition cages, exposure to adult whiteflies for 24−48 hours, depending on temperature. Whitefly adults were added by collecting from the eggplant in the whitefly colony using a battery powered electric vacuum device with a fine mesh organdy bag fit over the collection nozzle (Modified CDC Backpack Aspirator Model 1412, John Hock Co. Gainesville, Florida). Whitefly was collected for ca. 2−3 minutes and then transferred to cages at the rate of 0.12 L of adult whitefly per 20 plants.

After whiteflies were introduced, the plants were monitored over the next 1−3 days by collecting ten 2.54 cm^2 plugs per cage and counting the number of eggs with a dissecting microscope or by inspection of several intact leaves with a 10× hand lens. Once target density was reached, all adult whiteflies were removed by vacuuming, as described above.

After adult whitefly removal, plants were monitored every 2−3 days to remove any remaining whitefly adults, and any damaged or yellowing leaves, and wash off accumulated honeydew. Plants were monitored once a week for whitefly stage development by examining the leaves of several plants in a cage with a 10× hand lens. Whitefly nymphs typically reached the second instar, preferred by *Eretmocerus* spp. (Headrick et al., 1996), in 7−8 days during warmer summer months and about 12−13 days during cooler periods of the year. Once it was observed that approximately 50% of the whitefly nymphs were second instar or later stages, the cage was readied for parasitoid introduction.

5.5.4 Parasitoid production

Parasitoids were released as adult wasps or as parasitoid pupae inside the cages (Fig. 5.2A). To protect parasitoids from heat during transit they were carried to the rearing cages in Styrofoam ice chests cooled to 13°C−16°C. Cooling the parasitoids also slowed their activity, which made them less likely to fly to the roof of the cage away from whitefly-

infested plants. Adult parasitoids were placed on the soil at the base of the plants in 20-dram vials or in 100 cm Petri dishes so that they would fly or crawl up to the leaves as they gradually warmed. Parasitoids were released at the rate of 667 adults per plant using vials or Petri dishes that contained no more than about 1000 wasps per dish. Parasitoid pupae were released in 60 cm Petri dishes, which were also placed on the soil at the base of the plant at the rate of 1334 pupae per plant. More parasitoid pupae than adult wasps were released because emergence rates were less than 100%.

Cages were left undisturbed for one week after parasitoid introduction to avoid disrupting adult wasps searching for whitefly nymphs. After one week, several leaves from the upper and middle parts of the plants were inspected for adult parasitoid searching activity. If no parasitoids were observed searching, any adult whitefly that emerged from nymphs were removed by vacuuming. This was necessary to reduce feeding stress on the eggplant while parasitoids were developing. This procedure was continued on an average of 2−3 days until harvest. It was important to carefully balance the density of whiteflies with the rate of parasitism. When whiteflies oviposited too heavily on plants, lower than expected rates of parasitism occurred, and prolonged adult whitefly feeding caused the plant to drop leaves before parasitoid development was completed. For the same reason, it was important to grow healthy and robust plants by providing adequate amounts of fertilizer, controlling pests, controlling high temperatures and making sure plants were not stressed by either excessively dry or wet conditions.

Ten days after parasitoid introduction, plants were monitored for developing parasitoid pupae. Fourth instar whitefly nymphs were monitored with a 10× hand lens for signs of advancing degrees of parasitism, such as displaced mycetomes, development of an amber colored cuticle, parasitoid eye development, and/or the appearance of a margin on the developing parasitoid pupae within the whitefly (Roltsch, California Department of Food and Agriculture Website: http://www.cdfa.ca.gov/phpps/ipc/biocontrol/insects/19eretmocerus-lifestages.pdf). Older leaves, typically holding the oldest and most developed parasitoids, sometimes yellowed and dropped from the plant and were collected to begin the parasitoid harvest.

When the monitoring showed that >50% of the parasitized whitefly held mature parasitoid pupae on the remaining healthy leaves, harvesting started. This was determined by sampling ten 2.54 cm^2 leaf plugs from leaves in the middle of the plant and counting pupae with a dissecting microscope. However, some of the insectary workers were able to accurately determine the state of harvest readiness by inspecting several plants per cage using only a hand lens.

If the parasitoids were not ready for harvest, the plants were monitored every 2 days until parasitoids were fully developed. During the summer months, parasitoids were typically ready for harvest about 10−12 days after their introduction. During the winter months, harvest commenced 14−20 days after parasitoid introduction. At harvest time, all leaves from the plants in the cage were stripped, stacked one layer deep on paper towels, placed in large brown paper bags (30.5 × 17.8 cm opening by 43.2 cm deep), and transported to the laboratory in a large ice chest maintained at about 13°C−16°C. These bags were kept in the laboratory in an incubator maintained at 16°C until processing.

5.5.5 Parasitoid processing

Parasitoid pupae still inside the host were removed from eggplant leaves with pressurized water and separated from dead whitefly nymphs and exuvia using a water and air separation process. Leaves with parasitoid pupae were removed from the leaves with a spray gun with an adjustable cone-shaped nozzle (Triggerjet, 22650 Spray Gun™, conejet spray tip #5500-ppb; TeeJet, Spraying Systems Co., Wheaton, Illinois) attached to a sink using a washing machine hose modified with a threaded hose attachment, which delivered a fine spray of water under pressure. Pupae were washed from leaves, one leaf at a time, by placing the leaf over a piece of nylon organdy stretched over a 30.5 cm diameter metal kitchen colander and held tight with rubber bands. Parasitoid pupae separated from the washed unparasitized whitefly leaves were transferred to a 1 L glass beaker with ca. 0.5 L of water with approximately equal to 1.5 mL of liquid detergent (concentrated detergent, low phosphate, no perfume) to break the surface tension of the water, mixed well, and allowed to settle for approximately equal to 5 minutes. Because there is a buoyant cell of air within the whitefly exuvia around the developed parasitoid, parasitized whiteflies with fully developed parasitoid pupae float, while unparasitized whitefly pupae sink (Fig. 5.2C and D). Floating parasitoid pupae were removed from the top of the beaker with a small 6.4 cm (2.5 in.) diameter steel kitchen strainer and transferred to a sheet of dry nylon organdy stretched over a steel frame and allowed to dry (Fig. 5.2E). This stirring and straining process was repeated 3−4 times until no more parasitoid pupae were found floating on the surface of the water.

Pupae were air-dried on the laboratory bench at room temperatures (20°C−24°C). Parasitoids generally took 0.5−1 hour to dry, and drying was speeded by providing extra air circulation with a table fan set at medium speed.

Once the parasitoid material was dry, it was further processed to remove dry dead whitefly nymphs and exuvia that did not sink in the water separation process. Dried pupae were brushed into a 0.5 L paper container, adding enough pupae to cover no more than about 3–4 cm of the bottom of the container. Dead whitefly nymphs and exuvia (=scales) were much lighter than parasitoid pupae and could be removed by shaking the containers allowing scales to float from the container and then removing them by suction or blowing air. We used a 1.2 m wide power exhaust fume hood with the front sash set low enough to create enough suction to pull scales out of the container while being gently shaken. Other methods for removing dried scales could be used if an exhaust fume hood is not available, such as fans or vacuums to remove dried scales while shaking the parasitoid material. After processing, parasitoid pupae were stored until ready for use. These techniques resulted in very clean parasitoid material with very low numbers of whitefly pupae (Fig. 5.2F).

A sample of production data collected from 20 generations of *E. emiratus* reared between March and July of 1997 is presented in Table 5.1. These data show that parasitoid production averaged 236,000/ cage, with a range of 65,000–580,000/cage. For our production facility, this translated to19,000–172,000 parasitoids/m^2. Depending on the season, this level of production was achieved in 10–28 days. A greenhouse with better heating and cooling control would allow for uniform production toward the shorter end of this range. The increase rate of production averaged 6.5-fold the parasitoid inputs with a range of 0.8–24.6 (Table 5.1). Unparasitized whiteflies that remained in the cleaned product averaged 4.1% with a range of 0.5%–8% (Table 5.1). Factors that led to lower production included infesting plants with too few or too many whiteflies, late introduction of parasitoids (after whitefly nymphs had passed second instar), poor emergence of adult parasitoids, cool nighttime greenhouse temperatures (below 20°C), prolonged high

TABLE 5.1 Production statistics for *Eretmocerus emiratus*, a parasitoid of *Bemisia tabaci*, mass rearing on eggplant in 1997.

Harvest	Generation	Input	No. of produced/cage	Increase	Percentage of white fly
Date	(1000 s)	(1000 s)	factor		
3/24	4	24	206	8.6	1.6
3/31	5	84	299	3.6	0.5
3/31	6	50	196	3.9	3.0
4/2	7	29	154	5.3	4.0
4/11	9	60	65	1.1	3.6
4/8	10	35	113	3.2	8.0
4/14	11	20	371	18.5	1.6
4/17	12	95	81	0.8	1.5
4/21	13	20	490	24.6	1.2
4/21	14	31	331	10.7	2.3
4/24	15	35	129	3.6	–
4/24	16	44	172	3.9	–
4/29	17	75	114	1.5	2.4
4/29	18	60	133	2.2	2.0
5/1	19	35	66	1.9	1.5
5/5	24	76	499	6.6	1.7
5/6	25	110	580	5.3	3.2
5/7	26	25	347	13.9	6.3
7/7	45	25	186	7.5	11.9
Average			236.4	6.5	4.14

temperatures (above 35°C), and poor-quality plants infested with aphids or spider mites or with root or foliar plant diseases. Controlling these factors was a complex process that required attention to the maintenance processes of host plants, whitefly hosts and parasitoid populations. With the refinement of rearing techniques and gained worker experience, annual production increased. Production levels of exotic parasitoids for the years 1994–98 were 9.3, 5.4, 8.3, 30.9, and 47.6 million, respectively, allowing large numbers to be released in augmentative and classical biological control programs.

5.5.6 Storage of parasitoid pupae

The cleaned and separated parasitoid pupae were stored in paper food containers or plastic vials in an incubator maintained at 15.5°C–16°C before use in the field or shipping to cooperators. At these temperatures, parasitoid emergence was delayed safely for 3–5 days with little effect on parasitoid emergence. Longer storage times and lower temperatures decreased emergence rates and longevity of adult parasitoids (J. Gould, USDA-APHIS, Buzzards Bay, Massachusetts, unpublished data).

5.6 Final remarks

Three systems for production of *Bemisia* parasitoids in the genera *Eretmocerus* and *Encarsia* were described, one a smaller scale system for initial production and evaluation of the numerous cultures collected during the foreign exploration effort and two methods for larger scale production to conduct augmentative biological control demonstration projects in support of classical biological control by establishment of new species.

Efficient production systems depended on providing high-quality host plants that were free of pests, good environmental control, and careful control and monitoring of the whitefly host population. When these conditions were met the greenhouse-based production systems could produce millions of parasitoids per week with production levels as high as 172,000 parasitoids/m^2/generation. Similar results could be expected for other aphelinid parasitoid species. The methods developed for the *Bemisia* parasitoids should be adaptable for other parasitoids of whitefly species targeted by classical and/or augmentative biological control programs.

The classical biological control program directed against *Bemisia tabaci* biotype "B" (=Silverleaf whitefly) in the 1990s was one the largest and most comprehensive programs in the history of biological control. The foreign exploration program for natural enemies of *B. tabaci* was comprehensive, covering 30 countries, and more than 130 shipments of natural enemies were sent to quarantine facilities in the US between 1991 and 1998. Climate matching was used to match the affected areas in the US with locations within the native distribution of *B. tabaci* and was used to prioritize foreign exploration. The ARS European Biological Control Laboratory in Montpellier, France was extremely valuable to the biological control program; its staff engaged in nearly year-round exploration, which led to the discovery of many parasitoids for evaluation by US researchers.

Mass-rearing facilities were established in Tucson, Arizona; Imperial and Sacramento, California; and Mission, Texas. Hundreds of millions of *Eretmocerus* and *Encarsia* species were mass-reared for several years for release and evaluation in the areas affected by Silverleaf whitefly, which included the subtropical agricultural areas of the US and Mexico. Mass-rearing techniques improved dramatically over the course of the program, beginning with laboratory rearing in environmental chambers on whitefly-infested hibiscus plants, to heated, outdoor field cages with large pots of kale and eggplant, to highly managed greenhouses that used large-leaf eggplants and mechanical removal of parasitoid pupae. The substantial number of parasitoids available for release enabled a large-scale field evaluation of biological control as an integrated component of management programs. In retrospect, the Silverleaf whitefly biological control program clearly demonstrated the potential benefits of classical biological control in annual row-crop agriculture. The success of the program was made possible by a robust and productive mass-rearing effort of the key natural enemies. The authors hope that the information provided in this chapter will be useful to future researchers faced with mass-rearing aphelinid parasitoids at the scale needed for a large, multistate biological control program.

5.7 Production of *Tamarixia radiata* Watson parasitoid of *Diaphorina citri* Kuwayama

Diaphorina citri Kuwayama (Hemiptera: Liviidae), commonly known as the Asian citrus psyllid (ACP) is the most important phytosanitary problem for the world citriculture at present. Direct damage is caused by the injection of toxins by nymphs and adults while they feed on plant sap, and consequently causes deformation of leaves and shoots affecting

the trees for years. At the same time, the high infestations by *D. citri* favor the growth of fumagin due to honeydew excretions affecting the photosynthesis process of the trees (Chien and Chu, 1996). Indirect damage is related to the transmission of the causal agent of Huanglongbing, a disease caused by *Candidatus* Liberibacter spp., a gram-negative bacterium restricted to the phloem (Bové, 2006). Due to the economic importance of *D. citri*, pest control mechanisms have been generated and established to mitigate the effects of the disease, including biological control strategies by augmentation using *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae).

D. citri has more than 104 species of insects, mites and entomophagous spiders reported as natural enemies; however, *T. radiata* is the most important nymphal parasitoid of *D. citri*, due to its specificity, attributes as a biological control agent, and capacity to contribute to the management of its populations (Sánchez-González et al., 2015).

One of the objectives of a mass breeding system for beneficial insects is to produce the optimal number of individuals while minimizing production cost, work effort, and space, taking care of the quality of the final product (Arredondo-Bernal and Perales Gutiérrez, 2004). The mass rearing of any entomophagous insect requires three stages that involve the propagation of the substrate that supports the host species, the maintenance of the host population, and the maintenance of the beneficial species. To achieve the above, it is necessary to have the infrastructure and specific equipment, to know the biology (biological cycles, fertility, sex ratio, longevity), ecology, and behavior (preferences for some stage of the host, mating) of the parasitoid and its host, and factors that affect them (temperature, relative humidity (RH), photoperiod, and ventilation) (Arredondo-Bernal and Perales Gutiérrez, 2004).

In this section, we describe the breeding procedure and the most remarkable concepts that can be applied for the establishment of a mass production system of *T. radiata* for the biological control of *D. citri* under greenhouse infrastructure and under semicontrolled conditions, as well as a brief analysis of the breeding stock established in various parts of the world.

5.8 Diaphorina citri

5.8.1 Taxonomy

The genus *Diaphorina* can be recognized because its antennae are much shorter than the width of the head, forewings broader at the apex with a dark pattern and well-defined points, aedeagus with three segments, metabasitarsus with two spines (rarely one), and metatibia with an incomplete crown of evenly spaced apical spurs (Burckhardt, 1994).

D. citri presents morphometric variation in the width, length, and venation of the wings' genal processes, and in the coloration. However, the species can be recognized by having a genal process longer than the vertex along mid-line (slender). Forewing pattern consisting of each a band of confluent brown spots along vein *Rs* and along outer margin, the two areas are well-separated by a white gap in the subapical region of *Rs*, membrane whitish (Fig. 5.3D) (Burckhardt, 2007).

5.8.2 Origin and distribution

The ACP *D. citri* was first described in Taiwan by Kuwayama in 1908 (Halbert and Manjunath, 2004). Early work on the origin of *D. citri* suggests Far East Asia (East Asia region of India) (Mead, 1977); however, the most recent reconstructions based on host plant origins and historical information suggest an origin in India (Hall et al., 2008). The psyllid was first reported as a major citrus pest in India by Husain and Nath (1927) who, in disclosing the damage it caused, were the first to describe what is now known as Huanglongbing.

According to Hoffmann (1936), the first record of *D. citri* in China was in 1934. In the New World, *D. citri* was introduced within the last 25 years (Halbert and Núñez, 2004). However, there is an earlier report of *D. citri* from Brazil in the early 1940s (Costa Lima, 1942); but no one can corroborate this record and it is certain that established populations were not reported until much more recently. Worth mentioning, Huanglongbing was not observed in Brazil until 2004 (Hall et al., 2008). Actually, *D. citri* has spread globally where citrus fruits are grown through different human activities (Fig. 5.3C).

5.8.3 Ecology and habits

Adult ACPs are small (2.7−3.3 mm long) with mottled brown wings (Fig. 5.3D). However, it has been observed that the quality of the host plant can influence morphological, body color, and physiological variation. *D. citri* changes in body color have been related to differences in body mass and fecundity (Moran, 1968; Wenninger et al., 2009).

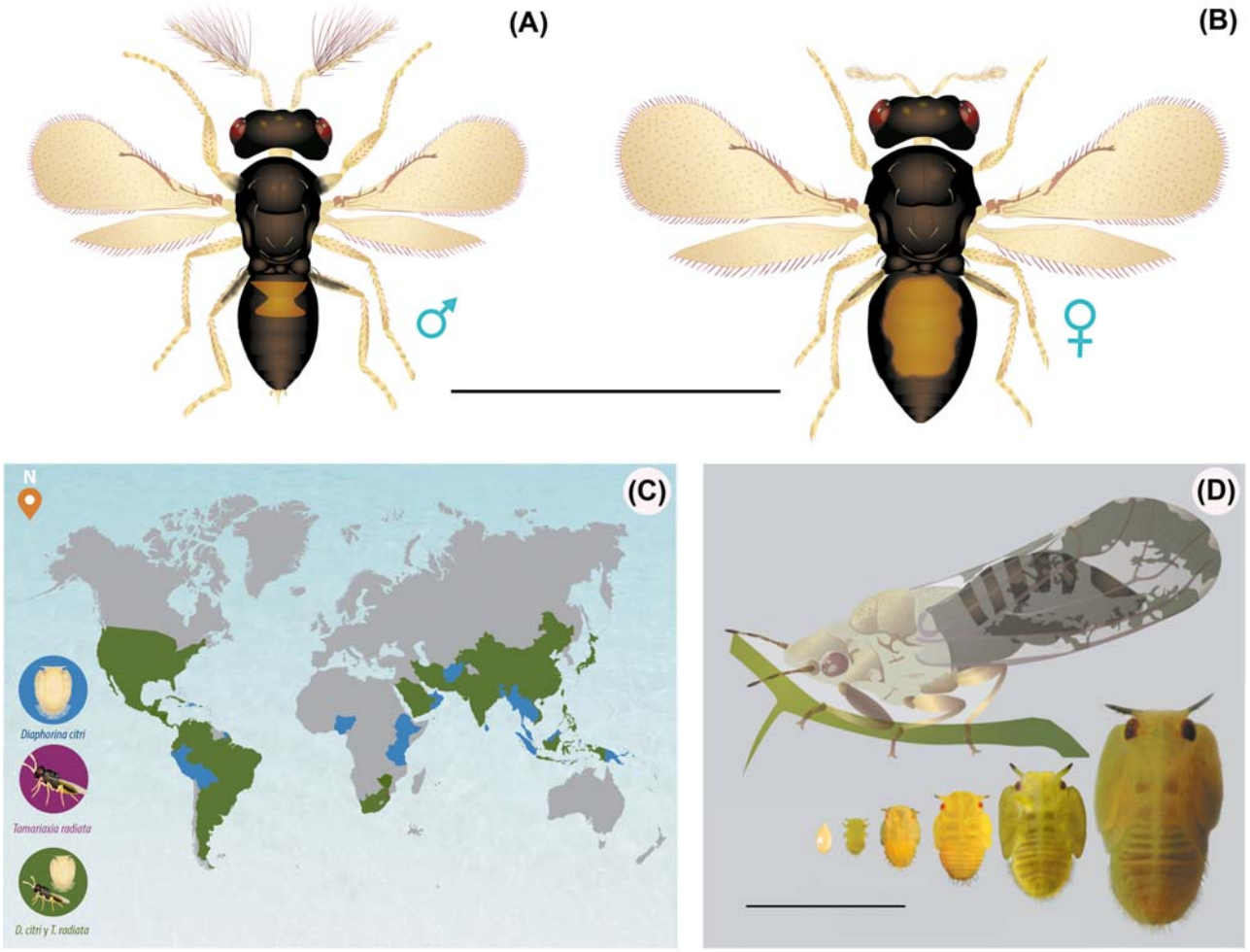

FIGURE 5.3 *Tamarixia radiata*. (A) Adult male. (B) Adult female. (C) Countries of distribution of *Tamarixia radiata* and *Diaphorina citri*. (D) *Diaphorina citri* adult and nymphs (from left to right: first to fifth instar). The scale line is 1 mm.

Morphological variation can also be attributed to abiotic factors such as temperature, humidity, and precipitation (Bomfim et al., 2011), photoperiod, and drought, among others (Hodkinson, 2009).

The adults are active, jumping/flying insects, they may be found resting or feeding on leaves with their heads at the leaf surface and their bodies held at a 45 degrees angle from the leaf surface (Hall et al., 2008) (Fig. 5.3D). The psyllid is a sucking insect and feeds on young stems and on leaves of all stages of development.

The psyllid's life cycle includes an egg stage and five nymphal instars. Nymphs feed on young leaves and stems (Tsai and Liu, 2000). Developmental times of eggs and nymphs vary with temperature: at 25°C, 17 days from egg to adult (Tsai and Liu, 2000), 49.3 days at 15°C, and 14.1 days at 28°C (Liu and Tsai, 2000). The optimal temperature range for *D. citri* is 24°C−28°C (Fung and Chen, 2006). Maximum adult longevity ranged from 117 days at 15°C to 51 days at 30°C (Liu and Tsai, 2000). Adults mate multiple times with different partners (Wenninger and Hall, 2008).

5.9 Tamarixia radiata

5.9.1 Taxonomy

A complete description of *T. radiata* is given by Waterston (1922) where he described *T. radiata* as *Tetrastichus radiatus*, and by Khan and Shafee (1981) who described it as *Tetrastichus indicus*. *Tamarixia radiata* is small dark nonmetallic wasps; male and female are similar in color and body structure, except for the type of antennae and a somewhat darker abdomen in the male (Fig. 5.3A). Males are slightly smaller than females in their overall length. Male's antennae are 1.5 times longer than those of the females (Onagbola et al., 2009) and have longer setae on the funiculum

(Fig. 5.3A). In addition, females have a broader club than the funicular segments (Fig. 5.3B), while in males the width of the club is approximately the same (Waterston, 1922) (Fig. 5.3A). As a genus of the subfamily Tetrastichinae, *T. radiata* has the maxillary and labial palps reduced to a single segment; the ventral edge of antennal scape of males with sensory plate; mesoscutum with notauli straight and complete, scutellum with two pairs of longitudinal lines and two pairs of setae (Fig. 5.3A and B); forewings with a dislocation of the submarginal vein at the level of the parastigma and absent postmarginal vein (Graham, 1987; Bouček, 1988; LaSalle, 1994).

5.9.2 Origin and distribution

Tamarixia radiata was initially described as *T. radiatus* in 1922 by the British entomologist James Waterston from specimens collected in 1921 in Punjab Province, northwest India (Waterston, 1922), currently part of Pakistan. The natural range of *T. radiata* extends from the Republic of Yemen and Saudi Arabia in the west to China and east to Indonesia (Parra et al., 2016). The encouraging results reported for the control of *D. citri* through *T. radiata* prompted both, the voluntary and involuntary global spread of this parasitoid (Fig. 5.3C).

5.9.3 Ecology and habits

Tamarixia radiata is an arrhenotokous, idiobiont, ectoparasitoid of nymphs of some species of Hemiptera, among them *D. citri* (Noyes, 2021). Its cycle comprises four life stages (Chien et al., 1991). The egg is translucent, ivory, and reniform; the larval stage consists of four larval instars, each distinguished by head capsule width (Chien et al., 1991). The mature larva ceases to feed, to give rise to the prepupal stage which secures the mummy to the plant surface by means of silken threads (Chien et al., 1991). Parasitoid pupae are yellow in color with hints of red. As the cuticle of the emerging adult fully sclerotize, adults chew a hole to get out of the mummy (Chien et al., 1991). The egg-adult development time varies according to temperature and photoperiod conditions. For example, at a temperature of 25°C and a photoperiod of 14 light hours, the parasitoid completes its cycle in approximately 12 days; with a duration of 2days in the egg stage, four in larvae, one in prepupa and 5 in pupa (Ching-Chin et al., 2001). However, there is a reduction in the development time of the adult egg to only 8 days when both the temperature (30°C) and the photoperiod (16 hours) are increased (Fauvergue and Quilici, 1991).

Males use their antennae to locate receptive females, which mostly mate once during the day of emergence; males can mate multiple times during their lifetime (Chien et al., 1991). Eggs can be laid after emergence, females inject venom into the host nymph through the ovipositor immobilizing it for 4−8 minutes (Chien et al., 1991) to deposit one or occasionally two eggs on the underside of the nymph, usually next to a mid or hind coxa (Chien et al., 1991).

Studies suggest that *T. radiata* prefers fifth instar *D. citri* for oviposition (Chien et al., 1991; Chu and Chien, 1991), although they also parasitize nymphs of the third and fourth instar (Chiu et al., 1988). Depending on the instar parasitized, there are differences in survival rates, body lengths of females and males, fecundity, and longevity, with parasitoids from the fifth instar of *D. citri* presenting higher values (Chien et al., 1991; Chu and Chien, 1991).

According to Chu and Chien (1991), there is a difference in the number of eggs deposited by a virgin or mated female of *Tamarixia radiata*, being 209.2 and 215.4, respectively. The sex ratio of the progeny is highly correlated with the age of the female parasitoid (Tang and Huang, 1991; Chu and Chien, 1991). The proportion of female offspring increased as the mother aged, from 0.5 for 1-day-old females to 0.77 for 22-day-old females (Chu and Chien, 1991). The sex ratio also correlated with the host stage.

Studies by Barr et al. (2009), González-Hernández. et al. (2010), INIFAP Instituto Nacional de Investigaciones Forestales, Agrícolas y Pesqueras (2013), Peña-Carrillo et al. (2014a,b) have recognized six haplotypes, that occur in different locations: (H1) USA (Florida and Texas), southern China, Taiwan, and Mexico (Colima, Hidalgo, Michoacán, Sinaloa, Sonora, Tamaulipas, and Yucatán); (H2) USA (Florida and Texas), China, Taiwan, and Mexico (Tamaulipas and Yucatán); (H3) USA (Florida); (H4) Vietnam and Mexico (Yucatán); (H5) Pakistan; (H6) Puerto Rico and Guadeloupe.

Tamarixia radiata has been successfully introduced to several countries to control *D. citri* and results for each country have been different (Lashkari et al., 2014; Qureshi et al., 2009). In Reunion, biological control with Pakistani *T. radiata* combined with the distribution of healthy plant material effectively reduced psyllid populations (Étienne et al., 2001). Good levels of biological control were reported in Guadeloupe by the introduction of *T. radiata* from Reunion Island (Étienne et al., 2001). Also, good levels of parasitism were recorded in the lower Rio Grande Valley of Texas by *T. radiata* from Pakistan (Flores and Ciomperlik, 2017); they chose to use individuals of *T. radiata* from Pakistan due to the similarity of the climate of Mulan, Pakistan, and the lower Rio Grande Valley of Texas (Goolsby et al., 2005).

However, when *T. radiata* from Taiwan and South Vietnam was released in Florida, the result was mediocre (Michaud, 2004; de Leon and Setamou, 2010).

It is possible that some populations of *T. radiata*, may be more effective for *D. citri* management when matched with pests that originate from the same region. It is here when the genetic analyzes that identify the different evolutionary lineages play an important role, by identifying the different haplotypes of the same species (Barr et al., 2009). Knowledge of the abundance, frequency, and dominance of haplotypes can be indicative of their adaptation to different environments. Use of the correct haplotype may improve parasitism or predation rates and effectiveness of natural enemies in biological control programs and therefore contribute to better pest management. Mass propagation and release of populations of biological control agents with greater possibilities of success in specific regions also reduce costs (INIFAP Instituto Nacional de Investigaciones Forestales, Agrícolas y Pesqueras, 2013).

5.10 Mass production

One of the main objectives of insect mass production from its inception to the present has been to develop a low-cost rearing system. A *Tamarixia radiata* biofactory must have a strategic geographical location, with access to electricity, water supply, flat land with a maneuvering cargo yard, and close to an airport if it is necessary to send parasitoid insects to remote destinations.

The mass-rearing process of *Tamarixia radiata* consists of three phases: (1) host plant production, (2) host production, and (3) parasitoid production (Sánchez-González et al., 2015). *Tamarixia radiata* grows exclusively on *D. citri* nymphs and, therefore, depends on efficient host rearing for economic viability (Parra et al., 2016). Rearing can be established in an infrastructure with controlled or semicontrolled conditions. The first requires air-conditioned rooms with sufficient lighting, which would limit production to small confinement cages. The method established by Skelley and Hoy (2004) proposed a rearing system conducted exclusively in environmentally controlled rooms that provide better control of species development and is ideal for experiments but is costly for mass rearing (Parra et al., 2016). The second option, which is the most commonly used by the insectaries established for mass rearing of *T. radiata*, uses greenhouse-type structures capable of controlling the temperature, providing a suitable environment for plant and insect biological cycle development inside the rearing cages.

In this section, we will review the experience of Mexico, which initiated the breeding of *D. citri* and *T. radiata* in January 2010, as part of a biological control program implemented by the National Service for Agrifood Health, Safety, and Quality (SENASICA by its acronym in Spanish) through the General Directorate of Plant Health (Sánchez-González et al., 2015), and over time has optimized rearing to produce an average of 750,000 adult specimens per month.

5.10.1 Infrastructure, equipment, and materials

The function of the greenhouses is to protect the rearing cages and maintain the temperature and RH under semicontrolled conditions. Their design is adjusted to the environmental conditions of the breeding site, taking as a reference the designs used for vegetable production, which can be covered by plastic or glass. The Laboratory for Mass Production of *T. radiata* from the Southeast (LRRTR, by its acronym in Spanish) in Yucatán, Mexico, has two greenhouses of 864 m^2 (24 m wide by 36 m long) each, with a rigid structure, covered by antiaphid mesh and with double access doors (Fig. 5.4A), in which 1000 cages are protected (Fig. 5.4B).

The greenhouses are equipped with industrial fans, air extractors, plastic curtains, shade nets, heaters, and dehumidifiers that mitigate sudden changes in temperature and humidity that help to keep the climatic conditions within the protected space as homogeneous as possible. As well as vacuum cleaners, air pumps, solar protection system with shade netting, irrigation system, and antiweed gravel on the ground cover, sanitary mats, and concrete walkways.

Cages can be made of stainless steel or metal (Sánchez-González et al., 2015), wood (Smith and Rohrig, 2016; Arredondo-Bernal et al., 2017), or polyvinyl chloride (PVC) (Skelley and Hoy, 2004; Parra et al., 2016). The LRRTR has metal structures of two sizes: the first is 0.7 m^3 cages and the second is rectangular 1.0 m high by 1.5 m long by 0.7 m wide. The tubular metal structure is divided by an antiaphid mesh and 0.5 m legs reinforced with galvanized PTR (Fig. 5.4C). The base of the structure that supports the plants is made of galvanized mesh and a double row of metal tubes to support an approximate weight of 120 kg (Fig. 5.4D). All tubular parts were painted with white enamel to prevent premature oxidation. The front of the rectangular cages has two aluminum doors for each partition, each supported by a hinge for opening and closing, with a plush snap clip on the edge of the door for a perfect seal. In total each

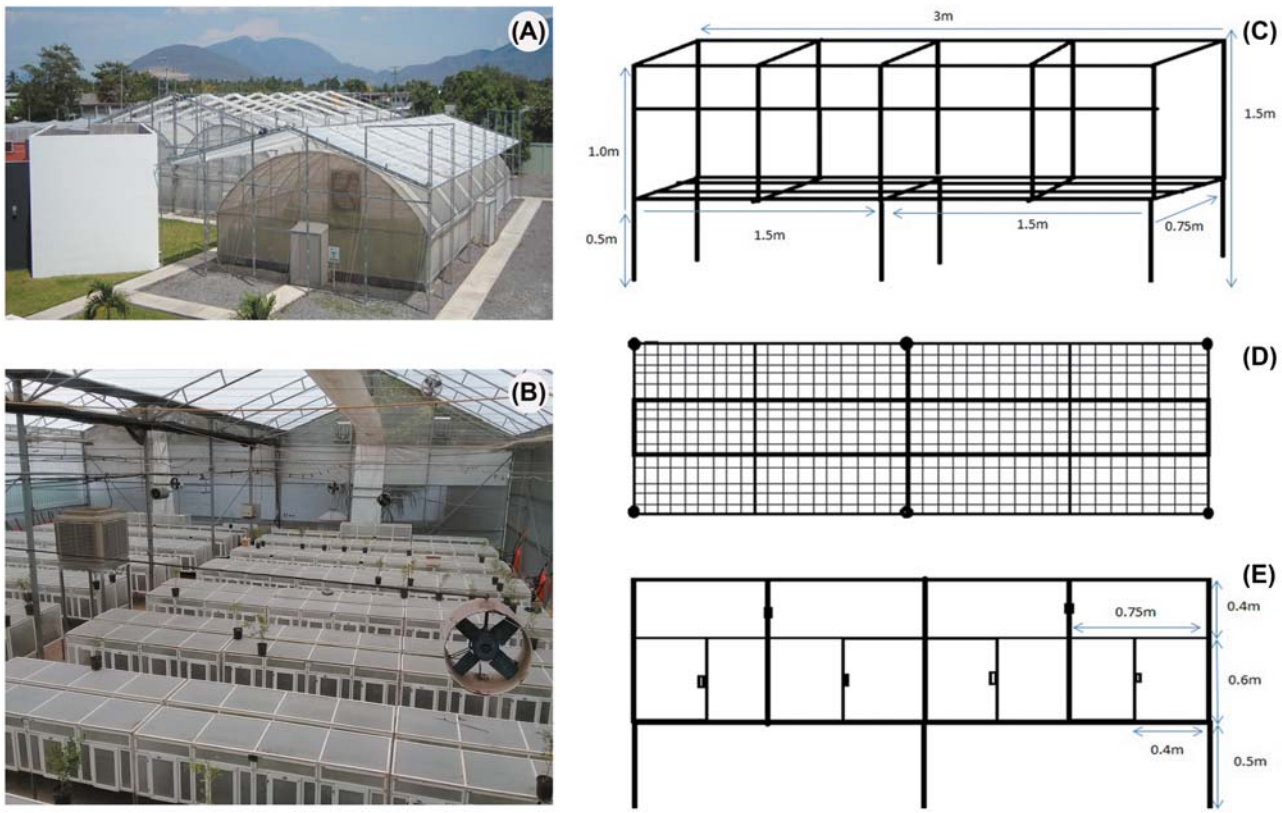

FIGURE 5.4 Greenhouse rearing facilities at the LRRTR, Yucatán, Mexico. (A) Exterior view of the greenhouses. (B) *Tamarixia radiata* rearing cages inside the greenhouse. (C) Design of the metallic structure for breeding cages. (D) Double cage structure base. (E) Front view of the double cage design structure.

structure has six doors; the lower doors are 0.4 m wide by 0.6 m high, while the upper door 0.4 m high by 0.75 m wide (Fig. 5.4E).

The surface of the structure is covered with white antiaphid mesh. Each mesh panel is fastened to the aluminum with a vinyl mouse-tail string, while the edges of the meshes are sealed with liquid silicone to prevent the escape of insects. Table 5.2 lists the elements required to operate a *T. radiata* mass-rearing laboratory under greenhouse conditions.

5.11 Host plant production

5.11.1 Characteristics, advantages, and disadvantages of using *Murraya paniculata*

Murraya paniculata (L.) Jack was originally described in 1920, belongs to the Rutaceae family, and with arboreal growth of 1.5−3 m in height with evergreen leaves that flowers all year round. Native to southeastern China, it is found in a wide range of geographic conditions from sea level to 1500 m a.s.l. (Gautam et al., 2012). It has bright green compound leaves, pinnately arranged and elliptically shaped. The flowers are terminal, corymbose and form aromatic white bouquets (Fig. 5.5A). The fruit is a fleshy red to orange berry with two embryos (Fig. 5.5B) (CONABIO, 2009; The Plant List, 2010). In the southeastern region of Mexico, fruit ripening occurs at the end of winter and in smaller quantities at the end of summer. *Murraya paniculata* is also known as orange jasmine, orange jessamine, china box, or mock orange, it has ornamental and medicinal uses and is also used as a living hedge.

Murraya paniculata is the most common host of *D. citri* (Halbert, 1999; Halbert and Manjunath, 2004; Skelley and Hoy, 2004); the oviposition rate of *D. citri* on *M. paniculata* is higher than on other citrus hosts (Nava et al., 2007). It is an easy plant to manage, low growing, thornless, and has rapid growth to generate new shoots compared to other species of the Rutaceae family, including the genus *Citrus* (SENASICA Servicio Nacional de Sanidad, Inocuidad y

TABLE 5.2 Elements for operating mass rearing of *Tamarixia radiata*, a parasitoid of Asian citrus psyllid, under greenhouse conditions.

Description	Use
Infrastructure	
Greenhouse-type structure, with white plastic roofs, covered with antiaphid mesh	They provide a confinement space with the possibility of favoring the environmental conditions for the development of the plant, the host insect and the parasitoid; as well as avoiding the entrance of organisms different from the breeding objective. The greenhouse floor is covered with gravel to prevent weed growth.
Ventilation equipment inside the greenhouse: fans, exhaust fans and heaters	They allow air movement inside the greenhouse, extraction of hot air and regulate the temperature.
Movable plastic side curtains on the outside of the greenhouse	They protect the rearing cages (which are located inside areas of the greenhouse) from rain, direct sunlight and wind. They are retractable.
Movable shade netting inside the greenhouse	Decreases temperature and light intensity by 50%.
Irrigation system	Provides water to plants for their growth and optimal development.
Electrical system	Facilitates the use of equipment or power tools.
Metal cages covered with antiaphid mesh 0.7 m^3	Plant confinement, Asian citrus psyllid and parasitoids for mass rearing.
Warehouse	For equipment and tool storage.
Air-conditioned room	To perform quality control, feeding and packaging of the parasitoid under conditions of controlled temperature and relative humidity.
Equipment	
Vacuum cleaner	To suck up remains of soil and leaves that fall into the cages when handling plants.
Pressure washer	Breeding cage washing to remove contaminants and impurities.
Bomb of void	To collect adults of *D. citri* and *T. radiata* that are inside the rearing cages.
Industrial dehumidifier	Lower the humidity inside the insect breeding areas. Fantech brand equipment 250 pints, with capacity to extract up to 115 L of water every 24 h.
PH meter	Measures the acidity or alkalinity of water or other solution.
Weighing machine	Weight of powdered or granulated fertilizers.
Electric hammer or drill	Repair of damaged cages.
Stereoscope Microscope	To perform quality control analysis of parasitoids.
Micrometer	To measure the length, width and tibia of *T. radiata* adults for quality control.
Double gooseneck LED illuminator	Illuminate the sample viewed on the stereoscope during quality control.
GPS	Coordinate location of the release points of *T. radiata*.
Freezer	Provide frozen gels for packing the parasitoids before transportation or shipment.
Computer	Store production data and quality control of mass rearing of insects.
Printer	Print receipts of delivery of biological material to users of the parasitoid, and other documents.
Materials in the parasitoid rearing area	
Table and chair	Organize the materials and equipment inside the greenhouse at the beginning of the day.
Trolleys	Allows plants to be transported within the greenhouse areas.
Entomological vacuum cleaners	Allow the collection of adults of *D. citri* and *T. radiata* in 110 mL flasks.

(*Continued*)

TABLE 5.2 (Continued)

Description	Use
Black cloth	Cover rearing cages, one wall of the cage will have access to light to gather insects on one wall and collect adults from there.
Pots	Plastic container, 2 L in volume, contains *M. paniculata* plants.
Pruning shears	Prune plant shoots.
Rakes	Collect leaves from pruned plants or move the gravel covering the soil.
Machete	To manually remove weeds from the greenhouse periphery.
Shovel	Used for digging the soil, turning soil and moving the substrate.
Hoses with watering can	To provide water metering for irrigation of the plants.
Rivet gun, ratchet, screwdrivers and metal lubricating oil	Used to repair damaged rearing cages, primarily rearing cage doors and greenhouse access doors.
Calculator	Facilitates the recording of production data per day.
Magnifying glass	Allows observation of immature stages of *D. citri*.
110 mL bottles with perforated lids	Contain the parasitoids or adult psyllids for the broodstock.
5 L unicel coolers	Mobilize jars containing *D. citri* or *T. radiata* within the rearing area.
Packing materials	
Aspirador entomológico	Herramienta para el control de calidad.
8 L unicel coolers	To confine vials with *T. radiata* that will be released in the field.
Brown paper	Cover the frozen gels and keep the 8 L unicel coolers dry.
Cardboard	Hold the frozen gels inside the cooler.
Honey	Feed the parasitoids during the journey to their destination.
Wax paper	Retain small drops of honey for feeding parasitoids.
Frozen gels	Condition temperature inside ice chests during parasitoid shipment.
Bottles 1 L	For observation of infested and parasitized shoots for quality control of the process.
Floral foam	Keep *M. paniculata* shoots turgid and moist during quality control.
Petri dish	Container for samples of insects, plant shoot or any other organism to be observed under the stereoscope.
Entomological tweezers and needles	To manipulate or hold the sample (insect, plant) under study.
Entomological pins and wooden tongues	For application of small drops of honey on waxed paper for insect feeding purposes.
Magnifying glass	Enlarge the sample to be studied.
Pruning shears	To cut shoots of *M. paniculata* for quality control.
70% alcohol	Preserve insect samples or plant shoots, also used for disinfecting the area.
Stationery	Recording production and quality control data.
Supply of pesticides and fertilizers	
Fungicides	They are used to prevent the growth of fungi such as *Capnodium* sp. or entomopathogens of *D. citri*.
Fertilizers	Granulated or foliar to improve plant development.
Insecticide	Used for insect control.
Substrate peat moos	It is used to improve soils due to its water retention capacity.

(*Continued*)

TABLE 5.2 (Continued)

Description	Use
Agrolite or perlite	Used to give porosity to the substrate and retain moisture for plant roots.
Soil	Base element to make the substrate mixture.
Protective clothing	
Plastic boots	Provides protection when washing *M. paniculata* plants.
Protective goggles	Provides protection at the time of chemical application and insect collection.
Latex gloves	Provides protection at the time of soil fertilizer application.
Leather gloves	Provides protection when handling any metal material.
Load belts	Prevent operator injuries when moving heavy objects (wheelbarrows loaded with plants or metal structures).
Industrial boots	Prevent injuries inside the production area.

FIGURE 5.5 *Murraya paniculata*. (A) Flower. (B) Fruits. (C) Seed drying. (D) Plants transplanted into 2 kg pots. (E) New shoots with symptoms of iron deficiency.

Calidad Agroalimentaria, 2015). Young shoots constitute a suitable substrate for oviposition and development of the first nymphal instars of *D. citri* (Baños Díaz et al., 2009). In addition, Skelley and Hoy (2004) considered that *M. paniculata* presented resistance characteristics to *Ca*. L. *asiaticus* and direct damage caused by psyllid feeding compared to citrus; however, Damsteegt et al. (2010) found that *D. citri* is capable of transmitting HLB to *M. paniculata* at a high rate. Therefore, it is recommended confinement during the entire plant development process using aphid netting cages and avoidance of contaminated psyllids.

5.11.2 Seed collection

At the beginning of the *T. radiata* mass-production program, an area for growing seed donor plants was included. These plants are planted directly on the ground to favor the complete growth of the bush; it is necessary to prune the branches periodically to stimulate flowering; pollination is essential for fruit formation.

In the absence of mature plants, *M. paniculata* fruits are collected from backyard plants through surveys in rural or urban areas of nearby localities (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015); harvesting is done during the winter in western and southeastern Mexico. The criteria for fruit collection are as follows (Colorado et al., 2017):

1. Select young plants in a productive state, with abundant fruit.
2. Select plants that present a good phytosanitary status, free of pests.
3. Plants with good architecture, leafy and green leaves, without visible symptoms of nutritional deficiencies.
4. Select seeds from a uniform harvest.
5. Select ripe fruits of similar size.
6. Do not collect fruits or seeds from the ground.
7. Harvest red or orange fruits with a slightly soft consistency.

5.11.3 Pulping and transport of fruit

The harvested fruits are transported in plastic containers with lids that allow ventilation to prevent fermentation (Colorado et al., 2017). If transport requires more than 48 hours, they are stored in expanded polystyrene (unicel) coolers with cooling gel. To obtain the seed, the fruits are placed in a container with water for a period of 12 hours, then drained and rubbed on a sieve or rough surface to allow the detachment of the pulp, and finally rinsed with plenty of water to remove pulp remains (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015).

5.11.4 Seed drying and storage

The seeds obtained from the pulping process (Fig. 5.5C), are placed on blotting paper for 24 hours to absorb moisture; this activity is carried out in a dry place with sufficient ventilation at temperatures of 20°C−25°C, avoiding direct solar radiation (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015), as it causes dehydration of the seeds and negatively affects the embryo due to the separation of the cotyledons. The paper should be changed every 12 hours to avoid the proliferation of pathogenic microorganisms that affect or inhibit germination (Wulijarni-Soetjipto, 1988); seeds with deformed cotyledons should be discarded during washing.

Perfectly dry seeds are stored in brown paper bags or in airtight plastic or glass jars; before confining them into the bags, it is recommended to spray a fungicide solution based on Captan at a dose of 2 g/L of water. The containers are labeled with the date and place of collection, fruit weight, and seed dry weight. Rice can be added to the paper bag or jar to act as a moisture absorber.

Since 2010, a protocol to safeguard or store the seeds in containers at quantities no larger than 1 kg at temperature between 20°C−22°C, was established. Seeds are renewed every winter, so seeds are rarely stored for more than 1 year. Approximately 10 kg (dry weight) of seeds are collected, which can produce 25,000−30,000 plants/year during the first 6 months of storage at a germination rate of 90%. González-Cabrera et al. (2016) determined that after 21 months of storage, using 25 g of silica gel per 100 g of dry seed at 5°C, germination rate is up to 53% ± 10%. It is recommended to carry out periodic inspections of seed containers to ensure that there is no physical damage or alterations by phytopathogenic fungi. In case of fungal infections, a powder fungicide application can be made (Colorado et al., 2017). In Belize (Lopez et al., 2014), it is recommended to plant the seeds as soon as possible after extraction and not to store them. Suitable conditions for seed germination are 30°C ± 3°C, 50%−60% RH, 30 m a.s.l. (SENASICA Servicio

Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015). It is advisable to do a viability test prior to each planting to determine germination rate to adjust the density of seed required for planting (González-Cabrera et al., 2016).

5.12 Production of *Murraya paniculata*

The whole agronomic process must be integrated as the production of an ornamental crop, preparing and stimulating the plant for sprouting, without the plant developing flowers.

5.12.1 Substrate preparation

The substrate for sowing and germination of the seed should provide porosity and humidity. The substrate for plant development used in the *T. radiata* Laboratory in Yucatan, Mexico contains 60% fertile local soil, 15% *Gymnopodium floribundum* Rolfe leaf litter, 5% cosmo peat, 5% compost, and 5% perlite; another substrate also used was clay loam-coconut dust-compost in a 3:1:0.5 ratio.

5.13 Sowing

When sowing in germination trays, it is recommended to place one seed at a depth of 1 cm/cavity. Irrigation should be done carefully three times a week with nebulization so as not to remove the seeds from the cavities. Seedbeds have a depth of at least 6 cm and allow root development once the seeds have germinated (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015).

The trays containing the seeds are placed inside the greenhouse on wire mesh tables. Sowing in germination trays offers advantages over sowing in seedbeds directly to the soil, this second method presents the risk of breaking the roots when the seedlings are removed from the soil.

5.13.1 Transplanting and watering

Plants 10 cm high and with the first sprout of compound leaves are transplanted to 2 kg pots where they will spend the rest of their cycle (Fig. 5.5D). The handling of young plants should be brief to avoid stress when removing them from the tray taking care not to break the root system. SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (2015) recommends transplanting early in the day. After transplanting, 100 mL of a mixture of a rooting agent at a dose of 5 g/L of water and worm humus at a dose of 5 mL/L of water is applied to each pot.

Manual watering is done three times a week or depending on the plant's needs and environmental conditions. Also, automated irrigation with a drip irrigation system directed to each pot is a viable alternative, but it is important to automatically control the irrigation regime when the plants are in the process of insect reproduction taking care not to saturate the plants with water during their vegetative development because water stress favors foliage loss and hinders vegetative recovery time.

5.13.2 Fertilization

During the first nine months of plant development, worm humus is applied to the substrate contained in the pot, and a hormonal product with different macro and micronutrients is applied to the foliage. After receiving fertilization treatment, plants should remain under 50% shade to favor greater sprouting and vigor (Parra et al., 2016).

The proper dosage of each fertilizer is based on what is indicated on the product label. It is essential to record the applications and determine the needs of the plants through the symptomatology of deficiencies or development (Fig. 5.5E). To decide which products to add to plant management, it is recommended to perform a soil analysis once a year and, based on this, correct the nutritional deficiencies of the plants and extend the time of exploitation in the insect rearing process.

5.13.3 Pruning

Pruning of *M. paniculata* plants serves as a stimulant to generate new shoots, ideal for the infestation of *D. citri*. The first apical pruning is performed when the plant is between 25 and 30 cm tall, the cut is made 5 cm from the apex of

the single shoot of the plant and it is expected to obtain at least three new shoots from this pruning (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015; Colorado et al., 2017). Pruning of plants that are in use consists of cutting long branches immediately after budding and the apical parts of compound leaves and old branches. Excess branches are also removed to clear for new sprouting. Each time pruning is performed, the plant should be disinfected with a fungicide to prevent the entry of pathogens (Colorado et al., 2017).

Murraya paniculata plants that have the following characteristics: (1) healthy with good bearing; (2) no weeds in the pot; (3) no insect contaminants; (4) with more than three shoots; (5) height of 30−40 cm; and (6) good root structure with white roots are transported to the *T. radiata* mass-rearing laboratory (Soper et al., 2014). A total of 10,000 plants in optimal conditions are required to supply 1000 cages for each production cycle of *T. radiata*.

5.13.4 Uses and reuse of plants

Each production cycle of *D. citri* and *T. radiata* is completed in approximately 28−35 days in each batch of plants depending on the rearing temperature. González-Cabrera et al. (2016) mention that *M. paniculata* plants were reused up to seven times; however, plant productivity decreases with reuses. Plants that are frequently used are pruned and consequently generate a greater number of shoots. After the first pruning, a plant produces three to four shoots per branch, while a continuously used plant produces five shoots, although up to 20 shoots suitable for oviposition are obtained with constant maintenance of pruning and fertilization (Parra et al., 2016). In LRRTR breeding, batches of plants are maintained and reused for 3 to 4 years. As time goes on the plant loses vigor and requires more attention for reuse. The total number of plants in use amounts to 60,000 specimens per year.

5.14 Host insect production

For mass rearing of *D. citri* it is necessary to have a batch of plants in reserve that is four times as large as the number of plants occupied per entry lot. During 2020, an average of 10,000 1 to 4-year-old plants per month and 200,000 18-day-old adult psyllids were used for the infestation process. The two types of rearing cages described in the infrastructure, equipment, and materials section were previously washed with a pressurized washer at 1800 psi and used to initiate mass production of *D. citri* for later exposure to *T. radiata*. The 1 m high cages allowed the plants to develop more freely while in confinement. A total of 10 plants per cage with the desired characteristics and with an optimal number of shoots for infestation measuring between 5 and 10 cm in length were selected (Palomares et al., 2015).

5.14.1 Environmental conditions for rearing

In the greenhouses of the LRRTR, average temperatures of 31°C and 60% ± 10% RH are maintained; however, maximums of 42°C and minimums of 15°C have been recorded. At the National Reference Center for Biological Control in Colima, Mexico, mass production of *T. radiata* was maintained at temperatures between 17°C and 35°C in winter and over 40°C in summer. Under these conditions, the highest population of the ACP was observed during the winter season (Palomares et al., 2015). In both cases, an automatic programmer activates the ventilation system when the temperature reaches 30°C.

The establishment of the *D. citri* broodstock is carried out under a protected greenhouse-type structure, equipped with double access doors, as described in the infrastructure, equipment, and materials section. It is also recommended to adapt windbreaks to the interior, between the access doors, to prevent the entry of flying insects (SENASICA, 2015) and ensure the availability of *M. paniculata* plants with young shoots before searching for insects.

Host *D. citri* is collected in the adult stage to avoid contamination with fungi and native parasitoids that commonly attack the nymphal stage (Arredondo-Bernal et al., 2017). The capture consists of shaking the branches of the plant infested with adults of *D. citri* to make the adults fall into a cooler (tapping technique), which allows them to be aspirated using an entomological aspirator. A total of 250 specimens are deposited in 110 mL vials. The foliage of *M. paniculata* is placed inside the vial so that the psyllids can perch and feed during transport to the laboratory. Vials are transported in coolers with refrigerant gel to avoid dehydration or stress on the insects due to high temperatures. A layer of paper or cardboard is placed between the gel and the vials to avoid direct contact with the cold surface of the gels and to prevent condensation inside the vials containing the adult psyllids.

Two breeding stands of field-collected *D. citri* are established using two different methods. The first method consists of maintaining a colony of the ACP in a series of frequently pruned and fertilized *M. paniculata* plants under greenhouse cover (SENASICA, 2015). This method allows constant and staggered sprouting in the plants, which favors the

development of multiple generations of the psyllid throughout the year while avoiding the presence of its natural enemies. The second method consists of retaining 20% of the total number of *D. citri* nymphs produced in the confinement for adult development. The total number of insects to initiate the colony is based on the number of shoots available on the plants; for both methods, 10 adults per shoot are introduced.

To ensure that the colony is genetically diverse, collections from different localities must be reproduced separately according to their place of origin (Soper et al., 2014). The offspring obtained from each cage are mixed for outcrossing and subsequently released into the rearing cages. In most organisms, the size and origin of the founder population seem to determine genetic flexibility and thus the probability of establishment (Mackauer, 1972).

5.14.2 Selection of adults for reproduction

Adults of *D. citri* for the next cycle reproduction are obtained from a production lot where 20%−30% of the total nymph population was not parasitized (Parra et al., 2016) and emerged as adults. At the time of the selection, these adult psyllids are approximately 15 to 18 days old. Cages are covered with black cloth to direct the adults of *D. citri* toward one of the ends that have been left uncovered by positive phototaxis, where they are collected and confined in groups of 250 specimens per jar at 1:1 sex ratio. The psyllids in the containers are allowed to mate while the cages where they will spend the rest of their cycle are being cleaned and conditioned. Each cage includes a label with the production lot date, infestation/parasitization date, number of plants, and average number of shoots. Selected plants should have developing axillary sprouts (brush shaped), so that the females oviposit and then the nymphs can spread throughout the entire length of the sprout, avoiding crowding. The females oviposit in groups, on the foliar base and the underside of the young leaves (Fig. 5.6A). The maximum number of eggs oviposited per female varies from 630 to 1900, depending on environmental factors and the host in which she is found (SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2019).

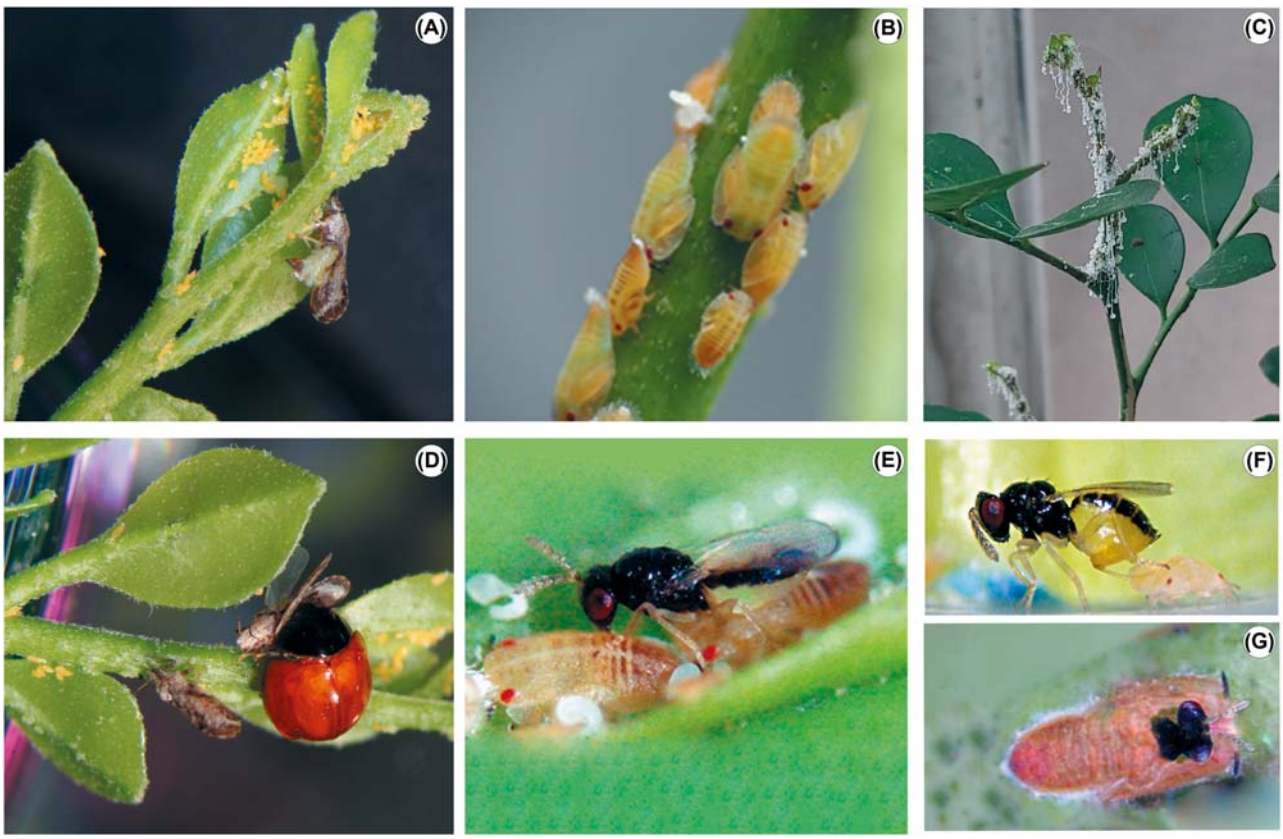

FIGURE 5.6 *Diaphorina citri*. (A) Female ovipositing on a *M. paniculata* sprout. (B) Nymphs without excretions. (C) Nymphs with excretions. (D) *Exochomus insatiabilis* feeding on adult of *D. citri*. (E) Adult female of *Tamarixia radiata* feeding. (F) Adult of *T. radiata* parasitizing nymphs of *D. citri*. (G) Emergence of *T. radiata* from *D. citri* mummies.

The biological cycle of *D. citri* varies according to the seasonal temperatures; in winter, the ACP cycle lasts 15 days with average temperatures of 25°C−28°C, while in spring it can be completed in up to 8 days with average temperatures of 38°C−40°C. Newly emerged nymphs cluster on the underside of tender leaves and have little mobility. Third instar nymphs migrate to leaf bases or branches, clustering in groups as a survival measure until adult emergence (García et al., 2016). An 18-hour photophase regime is recommended to promote greater oviposition (González-Cabrera et al., 2013).

For optimal parasitization, most of the host nymphs must be between the third and fifth instar, which is the ideal developmental stage for parasitism by *T. radiata* (Soper et al., 2014; SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015; Arredondo-Bernal et al., 2017). At this stage, it is important to control all environmental factors that may alter the development of *D. citri* lengthening or shortening the availability of suitable nymphs for parasitization to prevent more than 20% of the nymphs from escaping parasitism and emerging as adults. The white wax with honeydew excreted by the immature psyllids (Fig. 5.6B and C) falls on the lower leaves of the plant and can interfere with *T. radiata* parasitism. The honeydew takes the form of long tubules that emerge from the abdominal end of the nymphs and favor the development of fungi that cause fumagina (Halbert and Manjunath, 2004). The wax is removed by lightly tapping the base of the stem with a plastic utensil during the first hours of the day. In times of increased pluvial precipitation, it is recommended to perform this activity twice a day (morning and evening). If the wax is not cleaned in the morning during the warm season, the wax will run off in liquid form on the foliage, complicating its removal and damaging the developing nymphs.

Avoidance of undesirable introductions of native psyllid predators such as Coccinellids, which are the most persistent insect natural enemies, is highly recommended (Fig. 5.6D). Common native species include: *Olla v-nigrum* (Mulsant), *Hippodamia convergens* (Guerin), *Cycloneda sanguinea* (L.) (Sánchez-González and Arredondo-Bernal, 2010), *Curinus coeruleus* Mulsant, *Chilocorus* sp. (González et al., 2012), and *Exochomus insatiabilis* (Rodríguez-Vélez) (Rodríguez-Vélez, 2018).

5.15 Parasitoid production

5.15.1 Obtaining broodstock

The colony can be established in three ways: (1) Collection of adult individuals in orchards or backyards on plants of the Rutaceae family infested with populations of *D. citri* and naturally occurring *T. radiata*. The technique used to capture parasitoids is the "beating method," which is the same method above described to capture adults of *D. citri*. (2) Collection of *D. citri* nymphs parasitized by *T. radiata*, preferably when the parasitoids are in the pupal stage. It is important to observe the presence of meconium on the distal part of the nymph. Sprouts with parasitized nymphs are transferred to the laboratory (temperature of 25°C and 60% RH) to wait for the emergence of parasitoids. The collected sprouts are maintained inside a quarantine cage until adult emergence to avoid the introduction of other parasitoids or fungus to the mass-rearing area (Arredondo-Bernal et al., 2017). Adult parasitoids are aspirated and confined in a vial to facilitate their feeding by means of small drops of honey distributed on a strip of waxed paper. Adults from different localities are mixed before entering the mass-rearing cages to enhance genetic reinforcement of the founding colony. (3) Requesting *T. radiata* adults from other breeding laboratories.

The founding population in the LRRTR was 20,000 adult specimens. A large number of individuals used to start the colony is of vital importance to maintain a genetically strong population (Van Driesche et al., 2007).

5.15.2 Environmental conditions for breeding

Mass rearing of *T. radiata* at the LRRTR takes place in a tropical region at 20 m altitude, 31°C ± 10°C and 60% ± 10% RH. Breeding has also been reported under controlled conditions at a temperature of 25°C, 70% RH and a photoperiod of 14 hours photophase (Gómez-Torres et al., 2014). The increase in humidity (greater than 90%) can cause bud rot (anthracnose), a condition that favored the development of other contaminating fungi such as fumagina. Humidity control is accomplished by placing dehumidifiers in front of the cages where parasitoids emerge. Ventilation inside the greenhouse maintains the uniform internal temperature, and the airflow is directed to a distal end of the greenhouse where the exhaust fans are placed to expel hot air (Fig. 5.4B).

Newly emerged adult parasitoids are collected with vacuum pumps in doses of 20 females and 10 males per vial. Mating occurs in approximately 20 minutes. *Tamarixia radiata* has a daily oviposition rate of 11.26 eggs/female/day, with a mean generation time of 21.91 days. These data are used to calculate the number of generations per year (Baños Díaz et al., 2013).

5.15.3 Parasitization

Initially, infestation and parasitization were carried out in two different separated areas of the LRRTR. Plants infested with *D. citri* nymphs were moved from the infestation area to the parasitization area where they come in contact with the parasitoid. However, this method was simplified as one single procedure taking place within the same cage without the need to move the plant. As a result, rearing areas for *T. radiata* and *D. citri* are now the same. This practice saves time, labor, and space, making the process more economically sound.

Once *M. paniculata* plants have third to fifth instar *D. citri* nymphs, they are exposed to *T. radiata* (Soper et al., 2014; SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015), considering a ratio of 1:10 (i.e., one adult female *T. radiata* is released for every five *D. citri* nymphs) (Arredondo-Bernal et al., 2017). The age of wasps used for parasitization is from 24 to 48 hours. Wasps that feed on several ACP nymphs before parasitizing have been found to produce more offspring (Soper et al., 2014). It has been estimated that a single female of *T. radiata* can be capable of killing more than 500 psyllids by a combination of feeding and parasitism (Baños Díaz et al., 2013). *Tamarixia radiata* causes wounds on the thorax of nymphs to feed on hemolymph (Fig. 5.6E) and does not completely consume the nymph but leaves traces of entomophagy such as accumulation of hemolymph at the wound site, adhesion of its wing packets to the body, lack of dorsal symmetry, and nymphs are often attached to the leaf by solidification of the shed hemolymph due to dehydration (Vizcarra-Valdez et al., 2013b).

The parasitization process of *D. citri* nymphs is carried out in three phases: (1) 20 parasitoid females and 10 males are released in cages containing 10 plants with an average of three sprouts each and an infestation density of 50 nymphs/sprout, yielding 1500 potential nymphs to be parasitized; (2) on the third day after the first parasitoid release, a second release is made at a similar dose; and (3) on the fifth day another 10 females and five males are released. Parasitized nymphs are observed daily, and the honeydew excreted by the developing live nymphs is removed. Cages containing plants infested with *D. citri* that have just been subjected to parasitization should not be handled frequently and irrigation should be done with extreme caution. Any contaminants that threaten the development of the parasitoids should be removed. The parasitoid completes development in 12 days (Parra et al., 2016; Flores and Ciomperlik, 2017). On the seventh day after oviposition, the presence of meconium is observed in the parasitized nymphs and between days 11 and 12 the parasitoids are ready to emerge (Fig. 5.6G).

5.15.4 Collection of adults

Adult parasitoids are collected from the cages by taking advantage of their natural positive phototaxis. The cages are covered with a black cloth leaving the left and right-side walls uncovered, which allows the entrance of natural light. The design of the cages allows the collection of the parasitoids by a person in a sitting position (Fig. 5.7A). Parasitoids are captured with an aspirator connected to a vacuum pump using 110 mL vials at a density of 200 parasitoids per vial (SENASICA, 2015), It is advisable to direct the tip of the aspirator toward the head of the insects during aspiration to avoid damaging the wings during suction. Improper suction angles or collection speed can cause the adults to be crushed (Smith and Rohrig, 2016). Vials with *T. radiata* are placed inside a cooler with cooling gels to be transported to the storage and packaging room, where they will be checked, fed, and packaged for subsequent shipment to the field.

5.15.5 Handling and packaging prior to release

In the storage and packaging room, the containers with *T. radiata* from the greenhouses are kept at controlled environmental conditions of 22°C and 60% RH, where the following is performed: (1) Containers are visually inspected one by one to ensure that they contain only specimens of *T. radiata* (Fig. 5.7B) and biological contaminants, such as adults of *D. citri* or whiteflies, are removed with an entomological aspirator; (2) small droplets of honey are spread on strips of waxed paper 1 cm wide × 10 cm long and are vertically placed inside the containers to feed the adults (Fig. 5.7C). This food will help to prolong the life of the parasitoids for 48 hours while transported to the release destination. Bottles containing *T. radiata* adults are transported in coolers (Fig. 5.7D) containing cooling gels wrapped in brown paper and separated by cardboard strips to prevent condensation that could drown the parasitoids. Parasitoids can remain alive for up to 72 hours inside the cooler, shipment delays for longer than this period of time can result in the death of the parasitoids due to lack of food, lack of oxygen, and stress.

It is of utmost importance to establish quality control criteria to determine if the mass-produced natural enemies retain the adequate conditions to control the pest (van Lenteren, 2003). In the mass production process of *T. radiata*, parameters such as the average infestation of *D. citri*, the percentage of parasitism, the percentage of emergence, the

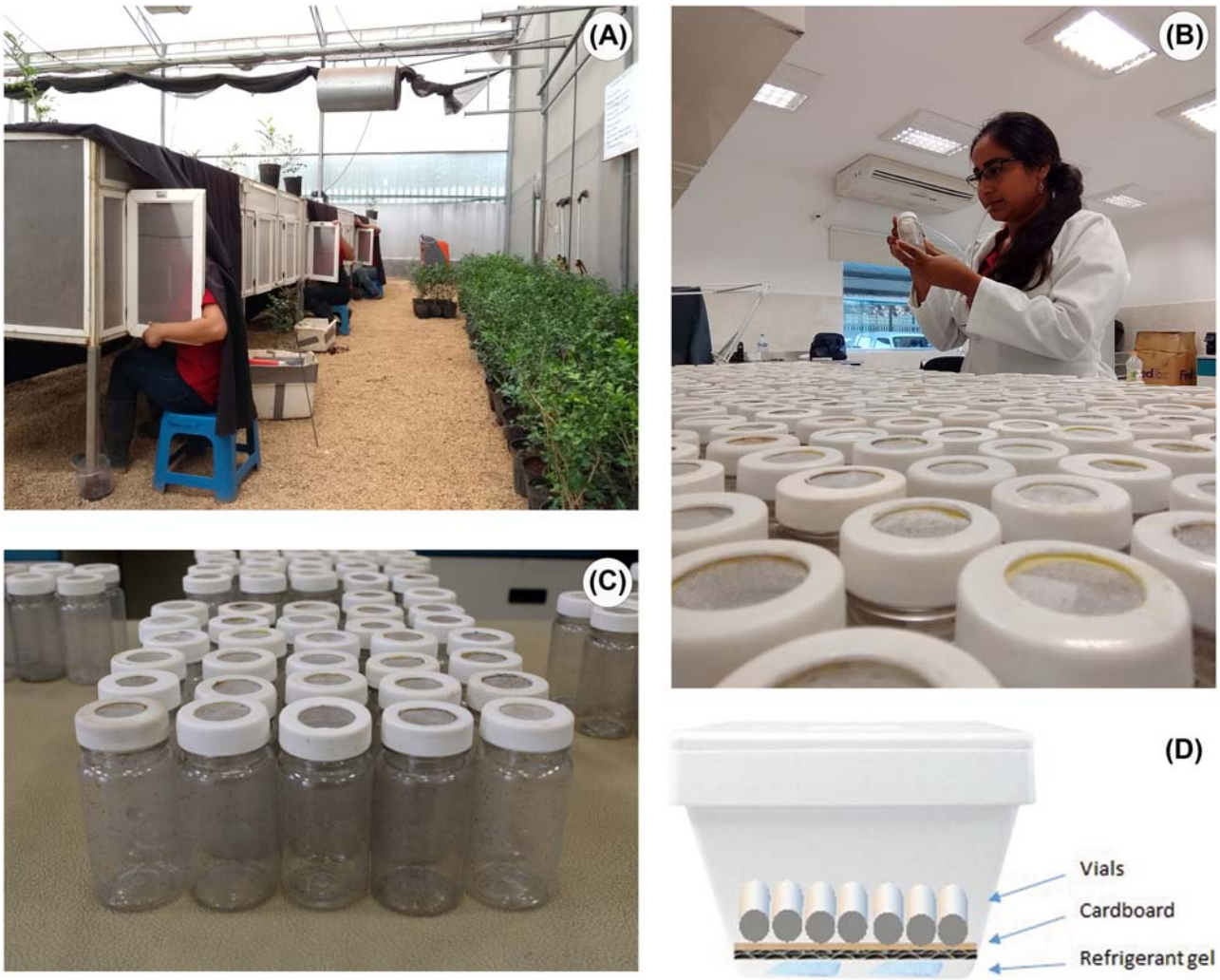

FIGURE 5.7 Parasitoid harvesting. (A) Collection of parasitoids by suction. (B) Visual inspection of flasks with *T. radiata*. (C) Vials containing adult parasitoids. (D) Packaging of parasitoids.

percentage of flying wasps, defective individuals, sexual ratio, fecundity, and measurement of body and tibia size are routinely evaluated (Vizcarra-Valdez et al., 2014; Baños Díaz et al., 2015; SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2015; Arredondo-Bernal et al., 2017; Poot, 2019). Indicators of the quality of *T. radiata* production should be applied to each production lot to continuously monitor the status of the colony and to record a history of the behavior of the lots used. The list of parameters evaluated during 2020 in Yucatán, Mexico, using the procedures established in SENASICA Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (2015) is presented in Table 5.3.

Analyzes of average nymphs (third to fifth instar) infestation levels per sprout in *M. paniculata* plants from 2018 to 2020 showed a regular fluctuation (Fig. 5.8A). However, in October 2020, the infestation levels dropped after the occurrence of hurricanes in the area and the colony suffered damage due to excess humidity. Parasitism dropped due to the low infestation levels to averages of 20% (Fig. 5.8), which forced the renewal of the broodstock.

Mass reproduction of *T. radiata* (haplotype 1) was established in January 2010 at the CNRCB and no evidence of deformities was detected between the start and completion date of this breeding stock (2017) (Vizcarra-Valdez et al., 2013a). Monthly production levels fluctuate between 100,000 and 1,350,000 wasps with that the highest number of *T. radiata* being obtained during the winter and spring months (Fig. 5.7C).

Insect colonies that are maintained as closed populations can suffer inbreeding, and consequently, gene variants that impact their biological fitness may be lost (van Lenteren, 1991). After nine years of mass rearing, no deterioration of

TABLE 5.3 Quality parameters in the mass production of *Tamarixia radiata*.

Parameter	Average obtained in LRRTR 2020	Acceptable average	References
Average infestation	59.6 nymphs/sproud	–	–
Percentage of parasitism	67.2%	60%–95%	Montesinos-Matías et al. (2020)
Percentage of emergence	95.1%	70.08%–95.23%	Palomares et al. (2015)
Percentage of flying wasps	100%	–	–
Defective individuals	0%	0%	Montesinos-Matías et al. (2020); Vizcarra-Valdez et al. (2014)
Sex ratio	2.06:1	2:1, female: male	Montesinos-Matías et al. (2020)
Fecundity	–	9 egg/female/day; 11.26 egg/female/day	Montesinos-Matías et al. (2020); Baños Díaz et al. (2013)
Longevity of females	22 days	23.1 days; 18 a 26 days	Montesinos-Matías et al. (2020); Baños Díaz et al. (2013)
Body size	1.19 mm	1.14 mm	Montesinos-Matías et al. (2020)
Tibia size	0.32 mm	0.24 mm	Montesinos-Matías et al. (2020)

the biological fitness of the ACP population and its parasitoid has been observed. However, the breeding stock has been renewed with adults of *D. citri* and *T. radiata* collected in ten different localities within a 50 km radius near the LRRTR, which consisted of adults adapted to the environmental conditions of the region.

5.16 Breeds of *Tamarixia radiata* established in other countries

Mass production of *T. radiata* has been established in several countries under the strategy of controlling *D. citri* populations distributed in urban areas, backyard citrus and difficult access areas, as well as abandoned orchards, and thus avoiding the migration of *D. citri* to citrus orchards established for commercial exploitation (Sánchez-González et al., 2015). The first breeding system for *D. citri* and *T. radiata* was developed by Ching-Chin et al. (2001) between 1983 and 1986, using *Citrus limonia* Osbeck as the host plant. Subsequently, Skelley and Hoy (2004) established a brood using *M. paniculata* in Florida, USA, as part of a classic biological control program. Both were developed in a quarantine facility under controlled conditions. The establishment of insectaries was initiated internationally using these production schemes with the objective of mass-producing *T. radiata* (Sánchez-González et al., 2015; Álvarez et al., 2016; Flores and Ciomperlik, 2017; Sosa et al., 2020; Paes-Bueno et al., 2020; Kondo et al., 2020; Sherwood and van Lenteren, 2020; Shogren et al., 2020).

Table 5.4 lists the countries that have the infrastructure to produce *T. radiata* and the institutions responsible for developing breeding for massive or experimental purposes in insectaries or laboratories. The United States (Florida, Texas, and California), Mexico, Brazil, Belize, Colombia, Costa Rica, Uruguay, and Argentina, have their production in greenhouse-type structures built of steel, aluminum, or wood materials. Barbados and the United States stand out because they have field breeding through the use of cages or devices with organza fabric that covers one or multiple citrus plants where they support the breeding of *D. citri* and *T. radiata* during few months of the year; in this case, the release of the adult parasitoid is passive through the opening of the cages.

Most insectaries use *M. paniculata* as a host plant for *D. citri* reproduction, only insectaries in California, USA and Colombia use *M. koenigii*. Uruguay and Costa Rica use *Citrus medica*, *C. limonia* and *C. sinensis* for the reproduction of *D. citri* and *T. radiata* in greenhouses (Table 5.4). Field cage production in California, USA, uses nine different species of common citrus in urban backyards (*Citrus volkameriana*, *Citrus limon* "Sicilian," *Murraya koenigii*,

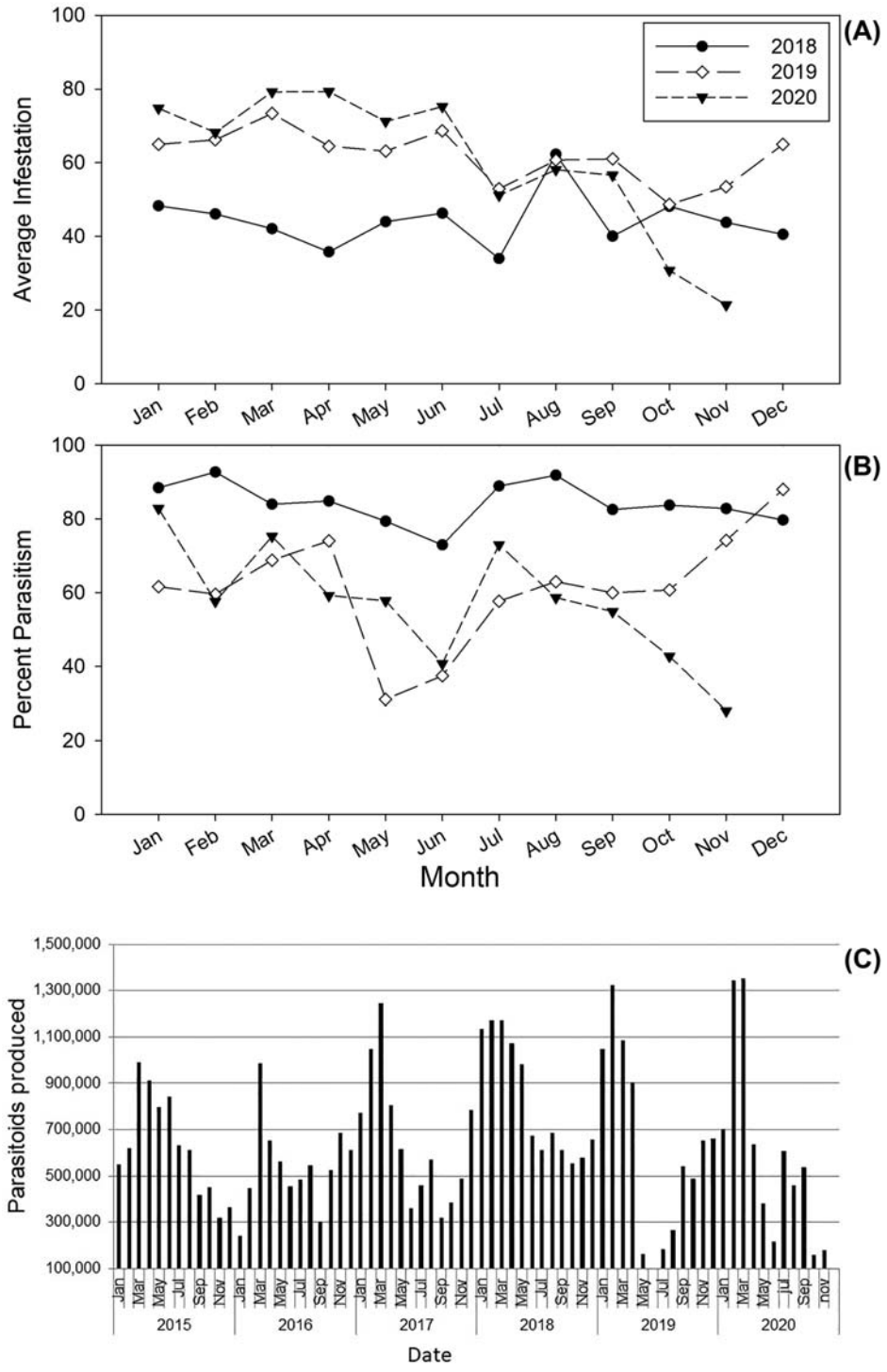

FIGURE 5.8 Parasitoid production. (A) Average infestation rate by *D. citri* of greenhouse host plants during 2018 to 2020. (B) Average percent parasitism by *T. radiata* in greenhouse reared *D. citri* during 2018−2020. (C) Parasitoid production between 2015 and 2020.

Citrus × *limon* "Yen Ben," *Citrus aurantium*, *Citrus maxima*, *Citrus aurantifolia*, *Citrus* × *limon* "Lisbon," *Citrus* × *sinensis*) (Shogren et al., 2020).

Some insectaries in the United States carry out mass breeding in greenhouses under controlled environmental conditions (temperature, RH, and photoperiod regulation). The rest of the production operations are in semicontrolled conditions, maintaining temperature and RH ranges with fans, hot air extractors, and humidifiers. The photoperiod is not

TABLE 5.4 Countries with massive and experimental rearing of *Tamarixia radiata*.

Country	Institution	Rearing			Host plant (hp)	Production under controled condiction			Adult parasitoid collection system	Production quantity (thousands)	Treated or protected area with biological control (thousand hectares)	Cost per specimen (USD)	References
		MASSIVE		EXP		HP	ACP	Tr					
		GH	Field (cages)	Lab									
First stage in the rearing establishment of *Tamarixia radiata*													
Taiwan	TARI/DAZ			1	*Citrus x limonia*	Yes	Yes	Yes	Entomological aspirator	UD	UD	UD	Ching-Chin et al. (2001)
Second stage in the rearing establishment of *Tamarixia radiata*													
United States: Florida	UF-SWFREC/ HSQ			1	*M. paniculata*	Yes	Yes	Yes	Mechanical vacuum cleaner	UD	UD	UD	Skelley and Hoy (2004)
Third stage in the rearing establishment of *Tamarixia radiata*													
United States: Florida	FDACS/DPI Gainseville	1			*M. paniculata*	Yes	Yes	Yes	Phototaxis + Mechanical vacuum cleaner	3300/year	UD	0.11	Álvarez et al. (2016); Smith and Rohrig (2016)
	FDACS/DPI Dundee	1			*M. paniculata*	Yes	Yes	Yes	Phototaxis + Mechanical vacuum cleaner		UD		
	UF		1		*Citrus* sp.	No	No	No	No aplica	UD	UD	UD	Qureshi et al. (2012)
Mexico	SENASICA-CNRCB	1			*M. paniculata*	S-C	S-C	S-C	Phototaxis + Mechanical vacuum cleaner	8400/year	266.4	¿?	Sánchez-González et al. (2015); Arredondo-Bernal and Rodríguez-Vélez (2020)

Mexico	Private Laboratory (Beneficial Insects of the North)	1			*M. paniculata*	S-C	S-C	S-C	Phototaxis + Panel with suction airflow + Mechanical vacuum cleaner	12000 up to 28000/year	UD	UD	Sánchez-Borja, pers. comm.
United States: Texas	USDA/ APHIS/PPQ & CPHST	1			*M. paniculata*	S-C	S-C	Si	Phototaxis + Mechanical vacuum cleaner	2000/year	UD		Flores and Ciomperlik (2017)
			1		*Citrus x limonia* & *C. aurantifolia*	No	No	No	N/A	12/tree	UD	UD	Flores and Ciomperlik (2017)
United States: California	UCR-I&QF			1 (16 strains)	*Murraya koenigii*	Si	Si	Si	Phototaxis + Mechanical vacuum cleaner	Up to 650/ year	UD	UD	Soper et al. (2014), Shogren et al. (2020)
	USDA/ APHIS/CRB	1			*M. koenigii*	S-C	S-C	Si	Phototaxis + Mechanical vacuum cleaner	Up to 1219/ year	UD	UD	Soper et al. (2014), Shogren et al. (2020)
	USDA/ APHIS/CRB		1		*Citrus* (9 spp.)	No	No	No	No aplica	Up to 120.9/ cage	UD	UD	Soper et al. (2014); Shogren et al. (2020)
	CDFA	1			*M. koenigii*	S-C	S-C	Si	Phototaxis + Mechanical vacuum cleaner	Up to 3485/ year	UD	UD	Soper et al. (2014), Shogren et al. (2020)
	FAR Inc. (private laboratory)	1			*M. koenigii*	S-C	S-C	Si	Phototaxis + Mechanical vacuum cleaner	Up to 265/ year	UD	UD	Soper et al. (2014); Shogren et al. (2020)
Brasil	MAPA/ EMBRAPA	6		1	*M. paniculata*	S-C	S-C	Yes	Phototaxis + Mechanical vacuum cleaner	~130/ month	12 (outside comercial citrus)	0.05	Paes-Bueno et al. (2020)

(*Continued*)

TABLE 5.4 (Continued)

Country	Institution	Rearing			Host plant (hp)	Production under controled condiction			Adult parasitoid collection system	Production quantity (thousands)	Treated or protected area with biological control (thousand hectares)	Cost per specimen (USD)	References
Belice	MA/OIRSA/ ICDF-Taiwan	1			*M. paniculata*	No	No	Yes	ND	UD	16	ND	Lopez et al. (2014); Sosa et al. (2020)
Colombia	AGROSAVIA	1			*M. paniculata* & *M. koenigii*	S-C	S-C	Yes	Phototaxis + Mechanical vacuum cleaner	20/month	1.5	0.18	Kondo (2017), Kondo et al. (2020)
Costa Rica	TICOFRUIT	1			*Citrus medica* & *M. paniculata*	S-C	S-C	Yes	Phototaxis + Mechanical vacuum cleaner	UD	6	UD	Camacho (2013); Blanco-Metzler and Morera-Montoya (2020)
Jamaica	RDD/MAF			1 (GH)	*M. paniculata*	No	No	No	Phototaxis + Passive collection	1/month	7.8	ND	Lee and Sherwood (2012); Sherwood and van Lenteren (2020)
Uruguay	UPERFRUY/ INIA/MGAP	1		1	*Citrus x limonia* & *C. sinensis*	Si	Si	Si	Mechanical vacuum cleaner	UD	UD	UD	Galván et al. (2017)
Barbados	MAFS/UF/ CABI		1		*Citrus* spp.	No	No	No	N/A	UD	0.3	UD	van Lenteren, Colmenarez (2020)
Cuba	CENSA			1	*M. paniculata*	Yes	Yes	Yes	Entomological aspirator	0.3/cage	UD	UD	Baños Díaz et al. (2015)

Argentina	INTA/ FONDAGRO/ FONTAGRO	1				Yes	Yes	Yes	Mechanical vacuum cleaner	500/year	150 1.5 (CBC)	UD	Mabel-Greco et al., (2020); INTA Instituto Nacional de Tecnología Agropecuaria (2020)
Proyectos por Iniciar													
French Guiana, Guadaloupe and Martinique	FREDON Guadaloupe	–	–	–	*M. paniculata*	–	–	–	–	–	–	–	Ryckeweart and Vayssieres (2020)

TARI/DAZ = Taiwan Agricultural Research Institute/Department of Applied Zoology; UF-SWFREC/HSQ = University of Florida/Southwest Florida Research & Education Center/High Security Quarantine; FDACS/DPI = Florida Department of Agriculture and Consumer Services/Division of Plant Industry; UF = University of Florida; SENASICA/CNRCB (acronym in Spanish) = National Service for Agro-Alimentary Public Health, Safety and Quality/National Center for Biological Control Reference; USDA/APHIS/PPQ & CPHST = US Department of Agriculture/Animal and Plant Health Inspection Service/Plant Protection and Quarantine/Center for Plant Health Science and Technology; UCR-I&QF = University of California Riverside-Insectary and Quarantine Facility; USDA/APHIS/CRB = US Department of Agriculture/Animal and Plant Health Inspection Service/Citrus Research Board Cooperative-Rearing Program; CDFA = California Department of Food and Agriculture; MAPA/EMBRAPA (acronym in Portuguese) = Ministry of Agriculture, Livestock and Food Supply/Brazilian Agricultural Research Corporation; MA/OIRSA/ ICDF = Ministry of Agriculture/International Regional Organization for Agricultural Health/International Cooperation Development Fund; AGROSAVIA (acronym in Spanish) = Colombian Corporation for Agricultural Research; TICOFRUIT = Costa Rican Orange and Pineapple Processing Company; RDD/MAF = Research and Development Division/Ministry of Agriculture and Fisheries; UPEFRUY/INIA/MGAP (acronym in Spanish) = Union of Producers and Exporters of Fruit of Uruguay/National Institute of Agricultural Research/Ministry of Livestock, Agriculture and Fisheries; MAFS/UF/CABI = Ministry of Agriculture and Food Security/University of Florida/Centre for Agriculture and Bioscience International; CENSA = National Center for Agricultural Health; INTA/FONDAGRO/FONTAGRO (acronym in Spanish) = National Institute of Innovation Transfer in Agricultural Technology/National Fund for Agroindustry/Regional Fund for Agricultural Technology.
HP = Host Plant; ACP = Asian Citrus Psillyd; EXP = Experimental; LAB = Laboratory; GH = Green House; TR = *Tamarixia radiate*; S-C = semi-controlled; UN = Information not available to the public or not generated.

controlled and the number of hours of exposure to natural light depends on the time of year. Literature mentions the existence of *T. radiata* production in China, however, no details were found in this regard, so it is not included in the list presented in Table 5.4.

The area protected by the biological control program through *T. radiata* in Mexico, Argentina, Belize, Brazil, Jamaica and Costa Rica is 266000, 150000, 16000, 12000, 7800 and 6000 hectares respectively (Arredondo-Bernal and Rodríguez-Vélez, 2020; Mabel-Greco et al., 2020; Sosa et al., 2020; Paes-Bueno et al., 2020; Sherwood and van Lenteren, 2020; Blanco-Metzler and Morera-Montoya, 2020). The University of California, Riverside, USA, maintains 16 genetically distinct populations of *T. radiata* in order to preserve the hybrid vigor of the parasitoid (Soper et al., 2014).

Mexico has the highest production of *T. radiata*, it has an official insectarium (dependent on SENASICA) and a private one, both of which produce 700,000(García-Cancino, pers. comm.) to 2.4 million *T. radiata* per month, respectively (Sánchez-Borja, pers comm.). The private laboratory "Insectos Benéficos del Norte" currently has the capacity to produce up to 28 million parasitoids per year (Sánchez-Borja, pers comm.); followed by the USA, Brazil, and Argentina. Declared production costs vary from USD 0.05 to 0.18 per individual (Table 5.4).

5.17 Production of parasitoids of muscoid flies

The market for filth fly parasitoids is fairly mature. Early successes in the 1970s led to long-running research efforts to identify the most promising parasitoid species and the situations where they can be most effective, especially on dairy, poultry and equine operations (reviewed in Machtinger et al., 2015; Machtinger and Geden, 2018). At least five commercial insectaries produce parasitoids in the US, with other insectaries operating in South America, Africa, Europe, and Israel. The most commonly produced parasitoids are pteromalids in the genera *Muscidifurax* and *Spalangia*, which differ in their life history and have somewhat different requirements for mass propagation (Fig. 5.9). *Nasonia vitripennis* is also available commercially but is generally not regarded as being effective for operational filth fly management.

Most of the common fly parasitoids can be identified using the excellent keys of Rueda and Axtell (1985) and Gibson (2000). The most commonly studied and commercially produced *Muscidifurax* species are one gregarious (*M. raptorellus* Kogan and Legner) and two solitary species (*M. raptor* Guirault and Sanders and *M. zaraptor* Kogan and Legner). *Muscidifurax raptorellus* is difficult to identify using morphological characters (Kogan and Legner, 1970) and is often recognized as appearing to be a gregarious *Muscidifurax* sp. (Petersen and Cawthra, 1995). *Muscidifurax* are characterized by relatively short development times (13−19 days at 27°C), high fecundity and attack rates, short adult lifespan, and a tendency to forage for hosts on or just under the surface of the fly larval habitat. The genus *Spalangia* is large and varied but the most commonly reared species are *S. cameroni* Perkins and *S. endius* Walker (Fig. 5.10). In contrast to *Muscidifurax* spp., *Spalangia* have relatively long development times (24−30 days), lower attack rates and fecundity, a longer lifespan, and will dig deeply into fly habitats to locate fly pupae. All of these species derive their adult nutritional needs from host-feeding and do not require the provision of honey or other sugar. They are ectoparasitoids that deposit their eggs in the space between the puparium and the pupa within, with larvae that feed externally on the developing/dead host inside the puparium.

FIGURE 5.9 Nearly all the filth fly parasitoids belong to the genera *Spalangia* (left) and *Muscidifurax* (right). *Spalangia* spp. are entirely black and include the common species *S. cameroni* (shown here), *S. endius*, *S. nigroaenea*, and *S. nigra*. *Muscidifurax* spp. have yellow legs, a yellow band on the anterior ventral abdomen, and include *M. raptor* (shown here), *M. zaraptor*, and *M. raptorellus*.

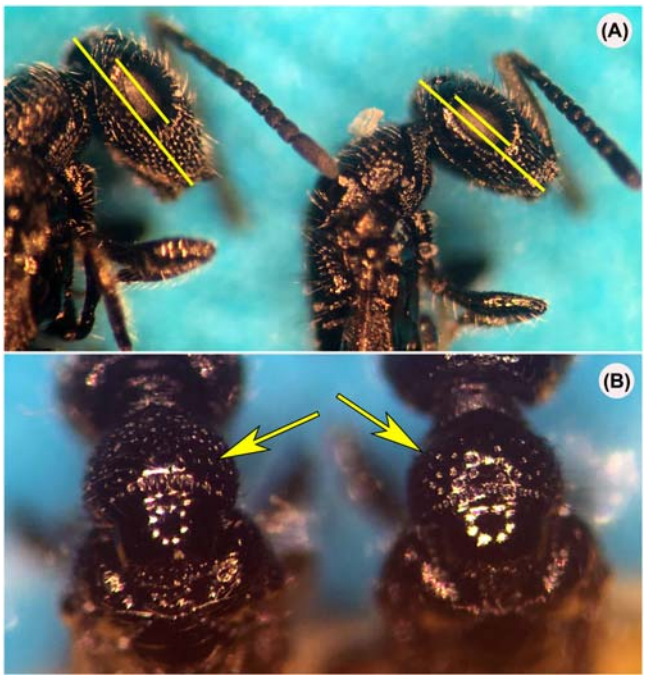

FIGURE 5.10 *Spalangia cameroni* (left) and *S. endius* (right) are the two most commonly reared *Spalangia* species. Two useful characters for distinguishing these species: (A) the size of the eye relative to total head length is smaller in *S. cameroni* than *S. endius* and (B) the dorsal pronotum of *S. cameroni* is densely covered with small hairy punctures, whereas in *S. endius* the punctures are larger and with more space between them.

5.18 Host production

The host range of pteromalid fly parasitoids is broad, and they can be reared on a wide range of fly species that are easily maintained in culture. Housefly (*Musca domestica* L.) is the nearly universal host of choice because it is easy and economical to produce on a massive scale. The following sections describe the rearing methods used at the USDA lab in Gainesville FL. Adult house flies can be maintained on any food resources that provide them with protein and simple carbohydrates. Our lab has long used an 8:8:1 mixture by volume of nonfat powdered milk, table sugar, and powdered egg yolk. Flies are maintained in metal screen cages ($38 \times 38 \times 45$ cm) that are set up with 6000 pupae and eight food dishes containing 60 cm^3 each of the above food mixture. This amount of food will last for the three weeks postemergence that the cage typically remains in production. Water is provided in 2-L buckets with foam packing "peanuts" placed on the surface that provide the flies with a dry perch when drinking. This method has a substantial advantage over wicking water systems. Because the packing peanuts remain as dry islands over the surface of the water, there are no suitable cues for egg deposition. In contrast, gravid females will readily oviposit on wet water wicks, which become slimy and laden with bacteria after several days of fly visitation.

Flies are ready to deposit their batch of eggs as early as 5 days after adult emergence. Although they are capable of producing >3 clutches of eggs, egg production drops markedly after two collections. Strong first oviposition is obtained by collecting eggs two weeks after the placement of pupae in the cage (ca. 9 days after emergence) at 24°C. An effective way to collect eggs is to wrap moist, spent medium (i.e., from which pupae have been collected) in black cotton fabric and place the pad in a cup in the cage. The cup with the oviposition pad is removed 3–6 hours later. Under conditions at the Gainesville lab, about 250,000 eggs are usually obtained from the first collection from a cage. Eggs are collected a second time one week later, with lower yields of about 200,000 eggs/cage.

As a precaution against *Serratia marcescens* becoming established in the colony, eggs are placed in a 0.4% sodium hypochlorite (bleach) solution, shaken vigorously to break up clumps, and left for 10 minutes. Eggs are rinsed twice with water and allowed to settle, then pipetted into graduated 15-mL centrifuge tubes to a volume of 2.5 mL, which is approximately 25,000 eggs. Each tube of eggs is used to set up one rearing pan of larvae. Eggs can remain in the water for 20 minutes with no loss of viability.

Housefly larvae can be reared in a wide range of media including animal manures, vegetable waste, and mixtures of grains. The larvae are obligate grazers on the microbial communities in the substrates and will not develop on sterile media. Several media-based mainly on grains have been developed that produce satisfactory results. The choice of media may depend on the availability and cost of ingredients and the scale of production.

TABLE 5.5 Amounts (kg) of dry ingredients needed to produce 15,000 housefly pupae using different recipes.

Ingredient	CSMA	Purina mix	Hogsette diet	Petersen diet
Wheat bran	0.6	0.6	0.9	1.3
Alfalfa meal	0.48	0.48	0.36	0
Brewers' grains	0.72	0.72	0	0
Calf Manna	0	0	0	0.36
Cornmeal	0	0	0.54	0
Cost per million pupae	$164.03	$881.83	$88.33	$89.54

Note: Water is added to dry ingredients for final moisture level of ca. 70%. Cost of ingredients are as of February 2021.

Table 5.5 gives examples of recipes and the relative costs for producing pupae. Changes in costs of individual ingredients have changed over the years. The CSMA diet was developed in the 1950s when spent brewers' grains were inexpensive. The cost of this ingredient increased in subsequent decades when it was recognized as a valuable commodity for feeding dairy cattle. The cost of alfalfa has also increased relative to wheat bran since the development of the CSMA diet. The Hogsette diet (Hogsette, 1992) was an effort to reduce rearing costs by eliminating brewers' grains, reducing the amount of alfalfa meal, and replacing some of the more costly ingredients with cornmeal.

The USDA lab in Gainesville currently uses the Petersen diet, which is composed of wheat bran and a pelleted calf feed (Calf Manna™) with water added to a final level of 70% moisture (3.8 L water with 1.66 kg dry ingredients). Both of the latter diets can be used to produce fly pupae at a cost of under $100/million pupae. A commercial premix (Purina fly diet from Land O' Lakes) that replicates the CSMA diet is also available when convenience is more important than cost. The amount of water added to the dry ingredients sometimes needs to be adjusted depending on changes in locally obtained ingredients or humidity conditions. For example, we find it necessary to increase water levels to 4–4.2 L in winter months when humidity in the rearing room drops below 40%.

Eggs (2.5 mL of settled eggs) are added to a rearing pan with one of the diets listed in Table 5.5. The pans are covered with a pillowcase to slow moisture loss, prevent unwanted oviposition by flies that are often loose in fly facilities, and stinging of newly formed pupae by stray parasitoids. Pupae usually can be harvested from the pans after 6–7 days at 27°C, although some strains may require as long as 9 days. Pupae can be separated from the medium by placing the medium in water, skimming the pupae from the surface, and repeating the process until pupae are as free of debris as desired. The wet pupae are then dried using fans (Fig. 5.11). This is the method currently used in Gainesville. Another harvesting method involves the use of forced air to drive the chaff and debris up and away from the pupae using a specialized device (Bailey, 1970). In yet another approach, water is added to the rearing trays as the larvae enter the wandering stage. This forces the larvae to leave the rearing pans and drop into a larger pan positioned below with a dry substrate such vermiculite, sand, or wood shavings. Pupal weights vary depending on the fly strain and the medium used are generally in the range of 18–24 mg/pupa. Pupae may then be stored in a household refrigerator for a week with no loss of viability. Keeping a log of harvest date, pupal weights, and yields from each batch is useful for quality control purposes. Slow development times, low yields, or changes in pupal weights can be signs that the amount of water added to the pans need to be adjusted, that the trays are being over- or underprovisioned with eggs, a problem with *Serratia* in the colony, or that the colony has been compromised by contamination with another fly strain.

5.19 Parasitoid rearing and housing

Fly parasitoids are produced by providing them with fly pupae that are replaced with fresh hosts 1–3 time/week. The pupae should be 1–2 days old and will remain vulnerable to parasitism for 2–3 days after placement. Housing methods and pupal replacement schedules depend on whether the colonies are being maintained at low-moderate levels for research or scaled up for mass production for field releases. Maintaining species purity is a top priority, especially when more than one species is being raised in the same lab for research projects. Faster-developing

FIGURE 5.11 Device for drying fly pupae after separating from media by floatation. (A) The drying box is constructed from plywood with an attached power supply and plastic containers with screen mesh bottoms are fitted inside the box. (B) Inside the box, two fans pull air through the top of the box and vent through the underside. (C) Wet pupae are placed in containers and covered with fine mesh screening (D) to prevent stray parasitoids from reaching the pupae.

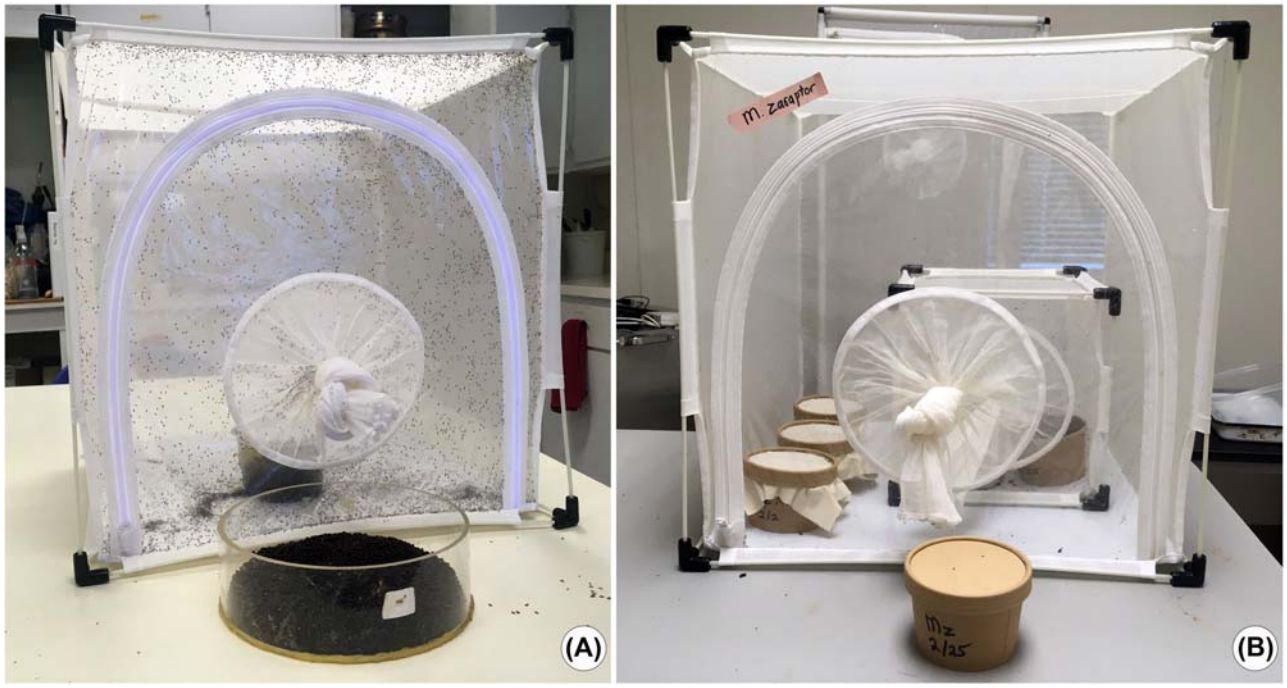

FIGURE 5.12 Examples of double (A) and single (B) containment for maintaining fly parasitoids. Double containment is necessary when multiple species are maintained in the same facility.

Muscidifurax species will drive slower *Spalangia* colonies to extinction within a few generations if they gain entry. In warmer weather, there is also a constant risk of *Nasonia vitripennis* entering the facility from outdoors. This species has a short development, is highly gregarious, and will out-compete species of *Muscidifurax* as well as *Spalangia*. The main point of entry for contaminant parasitoid species is via stinging pupae in the fly production operation. Newly harvested pupae must be protected from rogue parasitism at all steps in the stream from collection to placement in parasitoid colonies.

Smaller colonies can be further protected from contamination by using double containment. Fig. 5.12 shows an example of double containment using a small (21 cm cube) cage (BugDorm from Megaview Co., Taiwan) placed within a larger (45 cm cube) cage. Pupae (about 4000) are placed in the cage weekly in a 10-cm diam Plexiglas ring, depth 6 cm. with a no. 10 mesh screen (2 mm openings) bottom attached with hot glue. Pupae are removed by shaking the pupae in the ring to remove as many parasitoids as practical then transferred to paper cartons with cotton muslin covers. The cartons are placed outside the small cage but within the larger cage (Fig. 5.12B). Fresh pupae are placed in the ring and placed in the cage along with a batch of parasitized pupae with emerging adults. The oldest paper carton in the cage is retired and removed when the new group of emerging parasitoids is added. Colonies can be serviced weekly in this manner for curation/maintenance. Greater numbers of parasitoids for research projects can be produced by servicing the colonies 2−3 times/week.

Colonies for mass production at are maintained in a similar manner but on a larger scale. At the lab in Gainesville, parasitoids are housed in the larger cage and 10,000−30,000 pupae are placed in larger rings with screens (20 cm diameter, depth 6 cm) and changed 2−4 days per week. There are two approaches to stocking the cages with adult parasitoids. The first approach involves adding new adults to cages on an ongoing basis as described for small colonies. Greater control over host−parasitoid ratios can be achieved by stocking a cage with an estimated number of initial parasitoids. Estimates for the numbers of founders can be made by dissecting parasitized pupae to determine parasitism and adjusting the number of pupae placed in the cage to result in an estimated number of founding females. Another method for predicting the number of females in a cohort of parasitized pupae is to place a subsample of stung pupae at 28 °C−32°C and hold for adult emergence while the main batch of the cohort is held at c. 22 °C. Emergence and sex ratios in the cohort of pupae held at higher temperatures can be used to predict how many parasitized pupae. are needed to obtain a certain number of founding females.

An advantage of the method involving starting with known/predicted numbers of founders is that the number of pupae added to the cage can be adjusted to account for changes in the productivity of founders over the life of the cage. For example, sting rates for cages of *M. raptor* with 5000 female founders are strong and stable for the first 10 days but drop off rapidly thereafter in a 2-week production cycle (Geden et al., 1992). Host:parasitoid ratios of 2 hosts/female/day can be used for the first 10 days then adjusted downward to 1 host/female/day for the final sting on days 11−15. Using this schedule, 5500 founding females will kill 270,000 pupae and produce 116,000 female progeny over the life of the cage (Geden et al., 1992). With *S. gemina* (a close relative of *S. cameroni*), production efficiency is improved by stocking cages with lower levels (10,000 pupae) at the beginning and end of the two-week cycle and providing 20,000−30,000 pupae during the middle part of the cycle. The lower fecundity of this species relative to *M. raptor* means that 5000 *S. gemina* founding females will kill about 150,000 pupae and produce about 45,000 female progeny (Geden, unpublished data). Morgan (1986) provided a protocol for mass-rearing *S. endius* using higher parasitoid and host numbers that produced over 2.5 million parasitoids per cage per week. This paper is out of print and difficult to find but worth seeking for its details of a mass production system.

5.19.1 Host:parasitoid ratios

Choosing a host:parasitoid ratio for rearing fly parasitoids involves a balance between priorities. High host:parasitoid ratios produce the highest quality adults but are inefficient from a production standpoint because some of the pupae will escape host-feeding or parasitism altogether. A substantial number of exposed pupae, often called duds, will produce neither a fly nor a parasitoid. One cause of dudding is host-feeding without oviposition, which is most evident when high host:parasitoid ratios (e.g., 20:1 for *M. raptor* and *M. zaraptor* and 10:1 for *Spalangia* spp.) are used. In contrast, low host:parasitoid ratios result in superparasitism that drives up dudding rates because of competition among parasitoid immatures in superparasitized hosts. Dudding therefore imposes an upper limit on successful parasitism regardless of host:parasitoid ratios (summarized in Table 5.6). As a rule, 10,000 exposed fly pupae can be expected to produce about 3000−5000 females, depending on the species. *Muscidifurax raptorellus* is a notable exception because of its gregarious nature and will produce over 12,000 females from 10,000 exposed pupae.

5.19.2 Use of killed host pupae for parasitoid production

Live housefly pupae are only vulnerable to parasitism for a few days before they become too old for parasitism or produce an adult fly. This creates challenges when it is necessary to stockpile hosts for future needs. Pupae can be held at household refrigerator temperatures for a week with no loss of viability. Killed pupae can be held for longer-term storage, but their quality for parasitoids depends on the killing method and parasitoid species. Pupae that have been frozen are suitable for *Muscidifurax* spp. after several months of storage (Floate and Spooner, 2002; Kaufman and Geden, 2009). In contrast, *Spalangia* spp. do not develop well on frozen pupae, apparently because of moisture loss and host tissue decomposition during their relatively long development times (Floate, 2002; Kaufman and Geden, 2009). Gamma irradiation kills pupae without damaging them, and irradiated pupae can be stored for months with no loss of quality for

TABLE 5.6 Summary of parasitism by fly parasitoids under mass-rearing conditions.

Species	% successful parasitism	% females	Females per 100 pupae
Spalangia endius	80	67	54
Spalangia cameroni	57	67	38
Spalangia gemina	56	51	29
Muscidifurax raptor	70	69	48
Muscidifurax raptorellus	59	44	124

Source: Data compiled from Morgan, P.B., 1986. Mass culturing microhymenopteran pupal parasites (Hymenoptera: Pteromalidae) of filth breeding flies. pp. 77−78 in: Patterson, R.S. and Rutz, D.A. (Eds), Biological control of muscoid flies. Miscellaneous Publication Entomological Society of America. Vol. 62.; Geden, C.J., Steinkraus, D.C., Miller, R.W., Rutz, D.A., 1992. Suppression of house flies on New York and Maryland dairies using Muscidifurax raptor in an integrated management program. Environ. Entomol. 21, 1419−1426. and USDA and Cornell University rearing records.

parasitoids (Morgan et al., 1986). Heat shock is a more accessible option. Pupae that are killed by a 15-minute exposure to 55°C can be stored for months without loss of quality compared to live or irradiated pupae (Geden and Kaufman, 2007).

5.19.3 Disease concerns

Three pathogens can cause occasional problems in parasitoid rearing. The first is the fungal entomopathogen *Entomophtora muscae*, which occurs at epizootic levels in field populations of house flies (reviewed in Weeks et al., 2018). Infection is horizontal from one adult fly to another or contamination the environment by spore deposition. The introduction of new, wild fly stock from the field can result in the introduction of this pathogen, which is often followed by colony collapse about a week later. Cages with *E. muscae* contamination can be recognized by the large numbers of dead flies with swollen abdomens attach to cage sides and tops. The problem can be avoided by holding wild flies in a separate room for oviposition and then adding F1 pupae to the fly colony rather than adults. A second disease problem on the fly side of production is the bacteria *Serratia marcescens*, which often occurs at low levels in fly colonies without being detected. Symptoms of infection flareups in the colony include unexpectedly low pupal yields, long larval development times, the presence of reddish-colored dead larvae on the surface of larval media, and pink color in the water of egg suspensions when setting up new larval trays. *Serratia* problems can be held at bay thy thorough cleaning and disinfection of cages and rearing pans and by treating eggs with 0.4% sodium hypochlorite before adding to new larval pans.

Nosema disease is the main pathogen of concern in the parasitoids themselves. *Nosema muscidufuracis* in *Muscidifurax* spp. and related species in *Spalangia* spp. occur at low levels in field populations (Geden et al., 1995; Dry et al., 1999). Laboratory rearing conditions allow the pathogen to proliferate rapidly due to its ability to transmit vertically through transovarial transmission and horizontally by co-feeding of healthy and infected females. The horizontal transmission also occurs when healthy larvae consume microsporidia in superparasitized hosts that include infected larvae. Symptoms of infection include low fecundity/productivity, delayed development times, and sex ratios that are distorted somewhat in favor of males.

The most effective management tool for *Nosema* problems is to use the Pasteur method to prevent its introduction in the first place. Founding females from the field can be screened by allowing them to oviposit individually, examining them for infection (Cantwell, 1970; Fries et al., 2015), and discarding the progeny of infected females. If an infection has become established in the colony, remediation methods such as heat shock and drugs can be used to reduce infection levels to where the Pasteur method can be used to eliminate infection (Boohene et al., 2003).

5.20 Production of *Catolaccus grandis* (Burks) parasitoid of the boll weevil

Catolaccus grandis is an idiobiont ectoparasitoid of the boll weevil (*Antonomus grandis* Boheman) third instar and pupae. Females of *C. grandis* are synovigenic, which means that they have the capacity to continuously develop eggs throughout their adult life (Quicke, 1977). Parasitism of boll weevil larvae and pupae occurs in secluded environments like flower buds (cotton squares) or fruits. *Catolaccus grandis* is native to Mexico, where it is distributed from the Yucatan peninsula to the north part of the state of Veracruz in the tropical forest of the Gulf coast and the state of Sinaloa on the Pacific coast (Cross and Mitchell, 1969).

The first experimental releases of *C. grandis* in the US occurred in June of 1967 in Oktibbeha County, Mississippi and subsequent releases were done the following year in the same location (Johnson et al., 1973). Another attempt to establish *C. grandis* in the US was done in College Station and Brownsville, Texas during the summers of 1986, and 1987 (Cate et al., 1990). Although *C. grandis* did not become established, in all of the initial attempts substantial levels of parasitism were observed. Because of these promising results, new efforts in 1990 were directed to mass propagate and release *C. grandis* in cotton fields in the Rio Grande Valley of Texas with the objective of controlling the boll weevil using augmentative releases of this parasitoid. Field studies conducted between 1991 and 1993 documented successes in inflicting significant boll weevil mortality, reducing plant injury and preventing economical damage by the boll weevil in the experimental and commercial fields (Morales-Ramos et al., 1995a; Summy et al., 1992, 1994, 1995a,b, 1997; King et al., 1995; Vargas-Camplis et al., 1997, 1998). This series of successes represented the first records of a primary pest of an annual crop being biologically controlled by an augmented natural enemy. With the development of an artificial diet for *C. grandis* (Morales-Ramos et al., 1998; Rojas et al., 1996) and the subsequent release of in

vitro-reared parasitoids in experimental field (Morales-Ramos et al., 1995b), the boll weevil became the first example of a primary pest of an annual crop successfully controlled by augmentative releases of an in vitro-reared parasitoid.

5.20.1 In vivo production

The mass production of *C. grandis* became possible after two major breakthrough developments. The development of an artificial diet and mechanized mass production methods for the boll weevil (Sterling et al., 1965; Stewart et al., 1972; Griffin 1984; Linding, 1979, 1984; Roberson and Wright, 1984) and the development of artificial encapsulation methods for boll weevil larvae using Parafilm (Cate, 1987).

To encapsulate the boll weevil larvae, a Parafilm sheet was place on top of a perforated aluminum plate. A second aluminum plate with pegs that fitted inside the perforations of the first plate was pressed against the Parafilm sheet to create 8 rows of equally distributed capsules (Fig. 5.13A). One sheet of parafilm 6×24 cm produced 120 capsules. One boll weevil larva was introduced into each of the capsules and sealed with another sheet of Parafilm using a roller to fuse both sheets (Fig. 5.13C). This method simulated the natural environment of a boll weevil larvae infesting a cotton square. Parasitoids had no difficulties perforating the parafilm wall and ovipositing on the boll weevil host (Fig. 5.14A and B). Multiple eggs were oviposited on the inner walls of the parafilm capsules (Fig. 5.14C). First instars of *C. grandis* are highly mobile and able to find the host larvae and attach to it (Fig. 5.14D and E). This method was

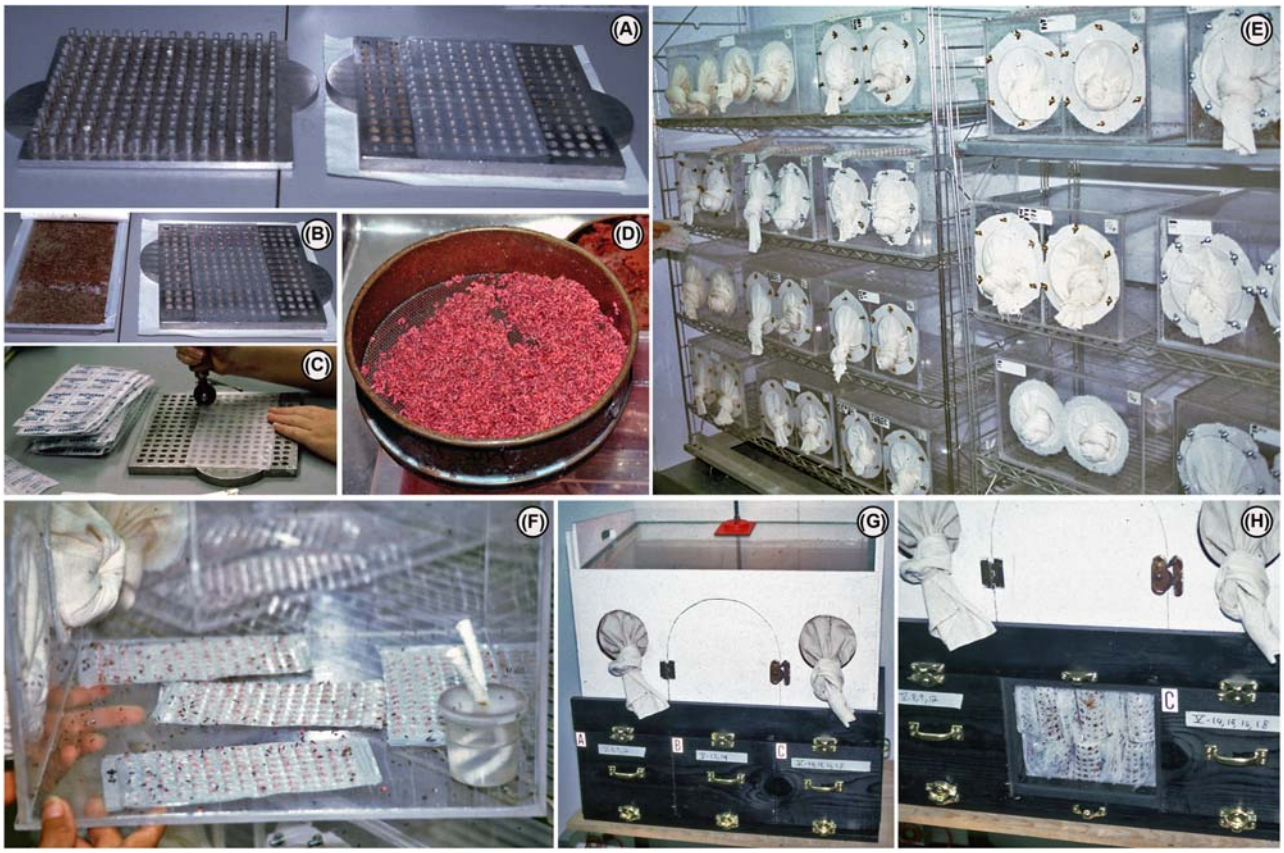

FIGURE 5.13 Basic hardware for rearing *Catolaccus grandis* in vivo. (A) Parafilm capsule forming press with rows of pegs (left) and wells (right) and a Parafilm sheet in place. (B) Boll weevil larvae are placed inside the capsules while still in the press. (C) Capsules are sealed with another parafilm sheet by pressing them using a roller. (D) Boll weevil larvae can be separated from the diet by washing them using a sift. (E) Adult holding cages in the main colony. (F) encapsulated boll weevil larvae are exposed to adult parasitoids by placing the sheets at the bottom of the cage. (G) Emergence cage consisting of a three-compartment emergence box at the bottom (Black) and a holding cage at the top (white). (H) Parasitized encapsulated boll weevil larvae (in rolled parafilm sheets) are placed inside one of the compartments of the emergence box when parasitoids reach the pupal stage.

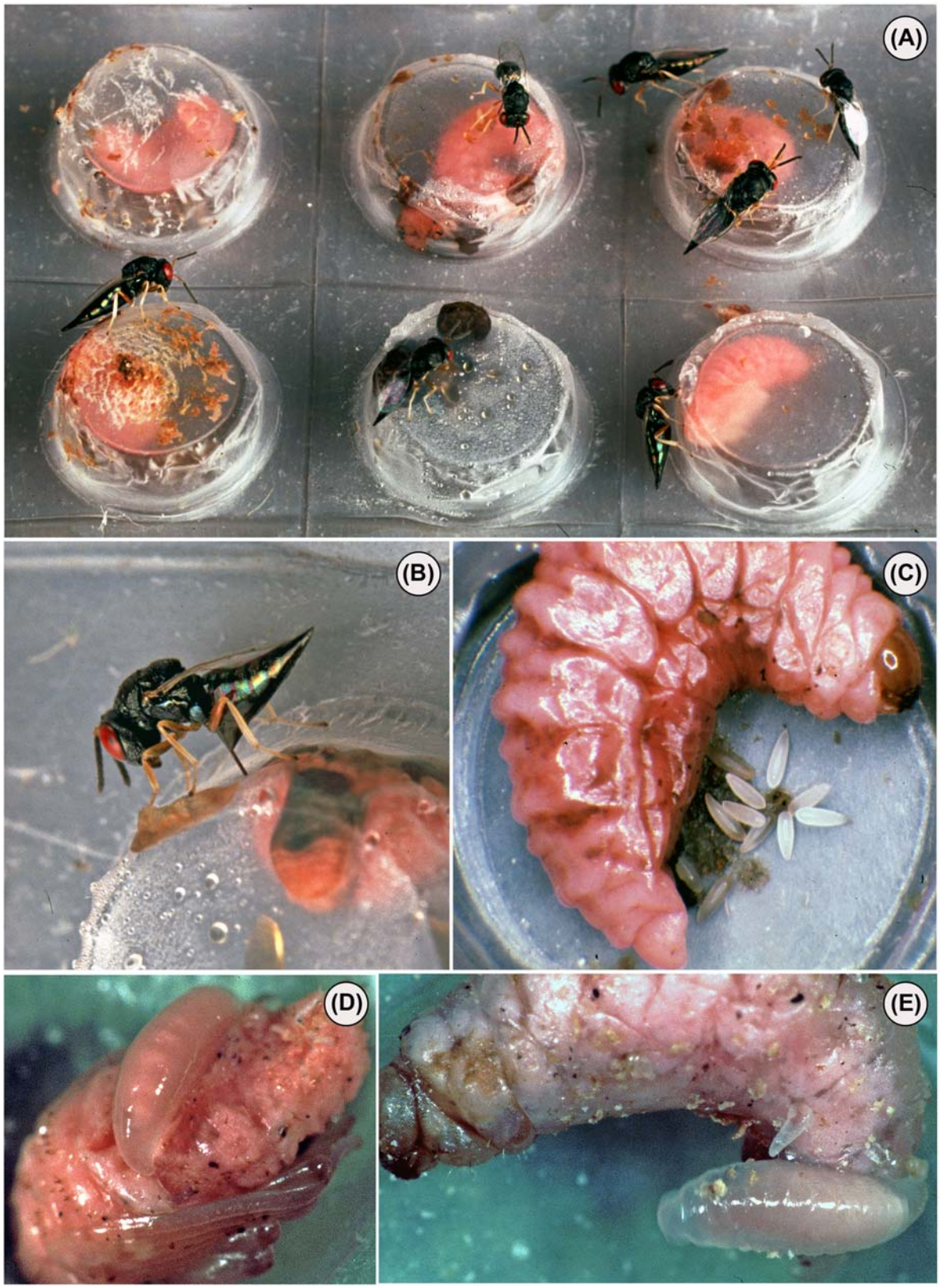

FIGURE 5.14 Boll weevil parasitism in Parafilm capsules. (A) Adult *C. grandis* females on encapsulated boll weevil larvae and pupae. (B) A female of *C. grandis* penetrating (and paralyzing) a boll weevil larva through the parafilm barrier. (C) Inside look of a capsule showing a paralyzed boll weevil larvae and parasitoid eggs. (D) *Catolaccus grandis* fifth instar parasitizing a boll weevil pupa. (E) *Catolaccus grandis* fourth instar parasitizing a boll weevil larva.

effective for rearing multiple species of boll weevil parasitoids including *C. grandis*, *C. hunteri* Crawford, *Bracon mellitor* Say, *B. compresitarsis* Wharton, *Heterospilus annulatus* Marsh, and *H. megalopus* Marsh.

The conventional in vivo rearing system of *C. grandis* consisted of oviposition cages (Fig. 5.13E) and two-level emergence box and cage systems (Fig. 5.13G and H). Adult oviposition cages were constructed of transparent Plexiglas with dimensions of $35 \times 35 \times 20$ cm (L × W × H). Two circular cut-outs of 10 cm diameter located on the front side served as a way to introduce encapsulated host into the cage and to provide water and honey to the adult wasps. The circular cut-outs were fitted with cotton sleeves to prevent wasps from escaping while the cage was being serviced (Fig. 5.13E). Each cage held a maximum of 500 adult females and between 100 and 500 males. Males usually died within a few days after introducing them to the cages. Females become sexually receptive 6 hours after emergence and mate only once becoming unreceptive approximately 1 hour after mating (Morales-Ramos, 1997; Morales-Ramos and Cate, 1992). Adult parasitoids were maintained at 27°C, 65% RH, and a 16-hour photophase (16:8 L:D), which were within the optimal ranges determined by Morales-Ramos et al. (1996a). Three sheets with 120 boll weevil larvae each were exposed to the female parasitoids for a period of 4 hours at 27°C (Fig. 5.13F). Longer periods of exposure resulted in high rates of superparasitism, reduced parasitoid survival, and male-biased sex ratios. Although normally two host exposures were done during a working day, up to three host exposures could be done per day without a significant reduction in the percentage of parasitism.

Parafilm sheets with parasitized host larvae were placed inside plastic storage containers and maintained in the dark at 27°C and 65% RH for a period of 8 days. At this time parasitoids reached the pupal stage and the parafilm sheets were moved to emergence cages. Emergence two-level box and cage system were constructed of wood with a glass top window (Fig. 5.13G). The bottom box was designed to hold the parafilm sheets containing parasitoid pupae in the dark (Fig. 5.13H). Emerging adult wasps chew through the parafilm to free themselves from the capsules. The bottom box had openings that connected with the upper cage to allow emerging adult parasitoids to move to the upper cage taking advantage of a strong positive phototropism. The opening holes connecting the emergence box and cage were fitted with 10 cm long plastic tubes, which were raised from the floor of the top cage to prevent parasitoids from falling back to the bottom box. The bottom box was divided into three compartments that allowed the placement of parasitoids from different batches. This resulted in a continuous process of adult parasitoid emergence. Parasitoid adults were aspirated and counted to place them into new oviposition cages in the correct density.

To prepare adult wasps for augmentative releases, females were exposed to a boll weevil host for a period of 5 days. This process was necessary to induce oogenesis (Morales-Ramos et al., 1996b) in the females and to get them reproductively active at the time of their release. Parasitoids were aspirated in groups of 250 females inside 1-L ice cream-paper-cannisters filled with shredded paper. The cannisters were opened in the field at a rate of I cannister per acre (4/ha).

A second-generation cage design integrated all these functions in one single three-level device that provides an emergence box at the bottom connected vertically to a holding cage (Fig. 5.15), which could be covered to force parasitoids into a release cylinder on top of the holding cage (Morales-Ramos et al., 1997). Parasitoids were maintained for a minimum of 5 days in the holding cage, where they were provided with honey, water, and encapsulated host. The adult females, already conditioned for egg production, were then forced to the release cylinder by covering the transparent cage with a dark clot. The release cylinder was filled with shredded paper like the regular release cannisters described above. However, unlike the release cannisters, the release cylinders were transparent, so the light could attract the adult parasitoids inside. The density of parasitoids in the release cylinders was regulated by placing the adequate number of parasitoid pupae in the emergence box. The average parasitoid survival was taken into account to adjust the optimal number of parasitoids that would end inside the release cylinder.

5.20.2 Factitious hosts

Catolaccus grandis is a selective parasitoid and its host range in the wild is extremely restricted (Cate et al., 1990). However, efforts were done to rear this parasitoid in a host that could be mass produced easier than the boll weevil. Parasitoids failed to develop on four lepidopteran species including *Galleria mellonella* (L.), *Helicoverpa zea* (Boddie), *Chloridea (=Heliothis) virescens* (F.), and *Chilo plejadellus*. More success was obtained when female parasitoids were presented with three coleopteran species including *Chalcodermus aeneus* Boheman, *Anthonomus eugenii* Cano, and *Callosobruchus maculatus* (F.). All three coleopterans were successfully parasitized by *C. grandis* and the parasitoids completed development on these three species (Rojas et al., 1998). The most suitable factitious host was *C. maculatus*, which was the most preferred of the three species by *C. grandis* females and 80.2% of the larvae completed development on this species (Rojas et al., 1998). *Callosobruchus maculatus* larvae stimulated oogenesis in naive *C. grandis*

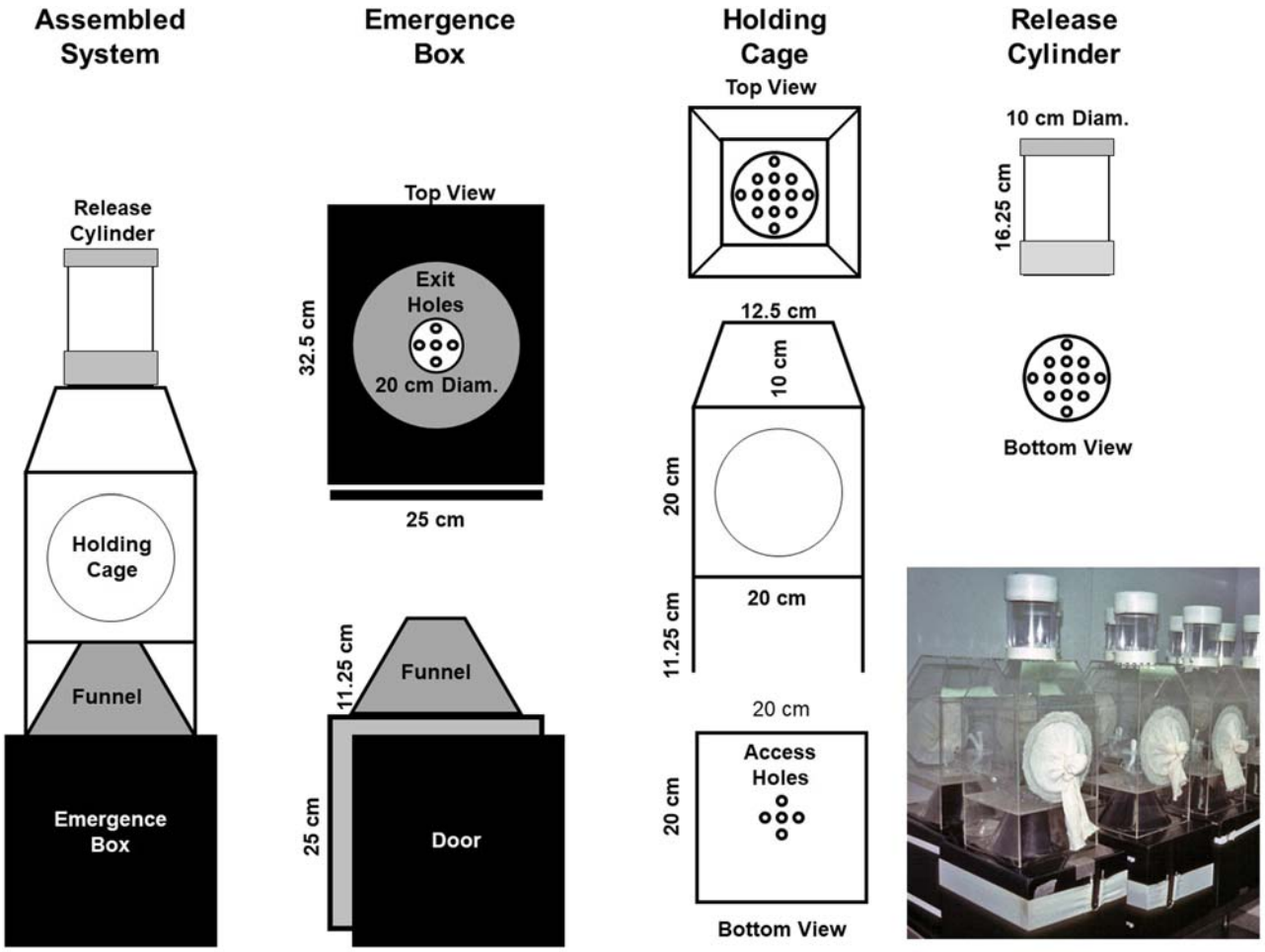

FIGURE 5.15 Diagram of the second-generation rearing cage for *C. grandis* showing the three components, which include the emergence box, holding cage, and release cylinder.

females when presented encapsulated in parafilm as compared with females with no host contact experience (Rojas et al., 1998). This suggested that *C. maculatus* could at least be used to induce oogenesis in parasitoids that were destined for field release.

Rearing *C. grandis* in *C. maculatus* for 10 generations did not show detrimental effects on the progeny, but it affected host preference significantly when *C. maculatus* larvae were presented as a choice next to the natural host, *A. grandis* (Rojas et al., 1999). *Catolaccus grandis* appeared to become adapted to the *C. maculatus* host over ten generations increasing its acceptance and preference as a host compared with the natural host. Nevertheless, *C. maculatus* performed well as a factitious host for *C. grandis* and provided a viable alternative to rear this parasitoid in vivo in the absence of a source of natural host rearing.

Callosobruchus maculatus was easily reared using garbanzo beans *Cicer arientinum* L. or black eye peas *Vigna sinensis* (Stickm). Seeds were broken in pieces between 2 and 3 mm before they were infested to facilitate the harvesting of the larvae for encapsulation in Parafilm. Otherwise, larvae would require to be extracted from the seeds one by one. The brocken seeds were presented to the adult beetles in trays for oviposition inside plexiglass cages with removable bottoms. Conditions were similar to those used for boll weevil rearing; 27°C, 60%−70% R.H., and 14 hours photophase. Larvae were harvested after 21 days using a sift standard No. 16. Rodríguez Leyva et al. (2002) determined that particle size impacted the size of the larvae and subsequently the size and fitness of the parasitoids; however, did not provide a reliable measure of the particle sizes tested. *Callosobruchus maculatus* larvae were exposed inside whole grains of garbanzo beans and successfully parasitized by adult females of *Catolaccus hunteri* Crawford (Vásquez et al., 2005). Parasitoids were able to parasitize the larvae inside the beans because third instars form a pupation cell inside

the beans and produce an unfinished tunnel that can be seen from the outside and presented a weak point for parasitoids to penetrate it with their ovipositor (Vásquez et al., 2005). This modality of presenting the factitious host to the parasitoids was never tested with *C. grandis*, but it may be a promising way to rear this parasitoid in factitious host because of its similarity with *C. hunteri*.

5.20.3 In vitro production

Production of *C. grandis* in vitro was possible after the development of a viable and effective artificial diet (Rojas et al., 1996). The original diet formulation (Gamma diet) was chemically defined, but several versions of the diet were produced during the refining process aiming at a meridic formulation with less chemically defined ingredients. The main objective of this process was to reduce the number of free amino acids added (initially 20 were added to the original formulation) and to eliminate the pure fatty acids, which were expensive and increased the diet cost. For instance, the delta diet had only 10 amino acids, but the latest version of the diet (Epsilon diet) included only five amino acids and no fatty acids in pure form (Table 5.7).

The Epsilon diet was evaluated, and parasitoids produced in this formulation were compared with in vivo-reared parasitoids using the methods described by Rojas et al. (1996) and Morales-Ramos et al. (1998). Parasitoids produced

TABLE 5.7 Formulation of the *Catolaccus grandis* artificial diet in mg per 100 g.

Ingredient	Amount (mg/100 g)		Ingredient	Amount (mg/100 g)
Amino acids			**Minerals**	
L-Glutamine	284.3		Calcium chloride	22
L-Histidine	183.4		Cobalt chloride	3.7
L-Proline	642.9		Ferric chloride	14.7
L-Tryptophan	330.2		Zinc chloride	3.7
L-Tyrosine	330.2		Potassium phosphate	54.1
Vitamins			Sodium phosphate	7.3
B_{12}	0.015		Magnesium sulfate	88
D-Biotin	0.044		Manganese sulfate	0.7
Ca-Pantothenate	1.47		Cupric sulfate	3.7
Choline chloride	0.147		Sugars	
Folic acid	0.007		D-Glucose	2,751.5
Myo-inositol	11.0		Emulsifiers	
Nicotinamide	3.7		Lauryl sulfate	82.5
Pyridoxine	0.22		Potassium hydroxide	617.4
Riboflavin	0.734		Preservers	
Thiamine	0.147		Phosphoric acid	5.5
Oligidic ingredients			Propionic acid	55.2
Egg yolk	16,417.5		Substrate	
Casein hydrolysate	1,009		Agar	256.8
Soybean peptone	917.2		Water	75,385
Brewer's yeast	160			
Olive oil	145.8			
Canola oil	210			

TABLE 5.8 Demographic parameters of *Catolaccus grandis* progeny reared in vivo from females reared in vitro using the Epsilon diet formulation compared with progeny from females reared in vivo.

Parameter	In vitro	In vivo
Net reproductive rate (R_o)	67.54	78.93
Intrinsic rate of increase (r_m)	0.175	0.185
Generation time (G)	24.05	23.65
Doubling time (DT)	3.96	3.75

in vitro were compared with those reared in the natural host by presenting groups of 12 encapsulated hosts per day to individual females held in Petri dishes (Morales-Ramos and Rojas unpublished). A total of 72 females per treatment were maintained in an environmental chamber at 27°C, 65% RH, and 14: 12 H (L:D) photoperiod. Parasitized hosts were kept under the same conditions until the parasitoid progeny completed development. The age-dependent fecundity, progeny survival, and sex ratio were obtained for each female using this method. This information was used to calculate life tables for parasitoids reared in natural host that originated from in vitro versus in vivo parental females. Demographic parameters for in vitro versus in vivo-reared parasitoids did not differ substantially (Table 5.8). Although females reared in vivo had a significantly higher pupal weight, 5.94 ± 0.15 and 3.85 ± 0.03 mg for in vivo and in vitro-reared females, respectively (mean ± SEM) ($F = 187.78$; df = 1, 142; $p < 0.0001$), the total number of eggs produced per female was not significantly different between in vivo and in vitro-reared females (363.07 ± 41.62 and 454.63 ± 30.91 eggs for in vivo and in vitro-reared females, respectively). The preoviposition period was not significantly different among treatments, but in vitro-reared females had a significantly longer longevity (23.67 ± 2.06 and 32.01 ± 1.67 days for in vivo and in vitro-reared females, respectively) ($F = 9.9$; df = 1, 142; $p = 0.002$). There was no significant difference in progeny sex ratio between in vivo and in vitro-reared females (2.13 ± 0.2 and 1.59 ± 0.25 females per male in the in vivo and in vitro-reared females, respectively). Progeny survival is difficult to determine in an in vivo system because of the high level of cannibalism, which mostly results in only one parasitoid developing per host. However, excessive levels of superparasitism can result in the death of all parasitoid larvae. Artificially placing one single parasitoid egg on a host larva is also unrealistic because *C. grandis* paralyzes the host during parasitism changing the host's nutritional value to favor parasitoid larvae nutrition (Morales-Ramos et al., 1995c). For these reasons, it is difficult to compare the immature survival of parasitoids developing in an artificial diet versus on a natural host.

The diet was presented in solid (agar-base) form inside multicell trays (Fig. 5.16A, H, and I). The diet was dispensed inside the cells while the tray was tilted 45 degrees to increase the surface available to the parasitoid larvae when the agar solidified (Fig. 5.16C). This process resulted in a small portion in the bottom of the cell that was free of diet. Parasitoid eggs were planted manually with the aid of a fine brush (Fig. 5.16F) or a micro vacuum manipulator at the bottom of the cell where it was free of diet to reduce microbial contamination. Parasitoid eggs were obtained by two methods: (1) from superparasitized encapsulated host larvae and (2) from host free devices consisting of Petri dishes covered with Parafilm impregnated with an oviposition stimulant (Fig. 5.16D and E). The oviposition stimulant consisted of a mixture of fatty acids resembling the ratios of the boll weevil larvae (Table 5.9) (Rojas et al., 1996).

Eclosing first instars moved to the diet leaving the egg corium behind. In a more advanced version of this process, a tray was specially designed with double cells, one larger than the other, to allow the placement of parasitoid eggs away from the diet (inside the smaller cell) without the need for tilting the trays at the time of diet dispensing. This double cell system takes advantage of the ability of *C. grandis* first instars to move relatively large distances to find the host. Larvae inside the double cell system moved quickly to the diet after eclosing. Dispensing the diet while the tray was placed horizontally facilitated the use of mechanized diet dispensing systems and provided the potential to mechanically dispense the parasitoid eggs inside the capsules (although this latest method was not developed). Survival of immature parasitoids from egg to adult in the Epsilon diet was 88.36% using this system. One disadvantage of the in vitro system was that the covers used to seal the cells were made of a material that adult parasitoids were unable to brake with their mandibles. This prevented them from successfully freeing themselves from the cell after emergence and the pupae must be removed artificially from the cell before emergence.

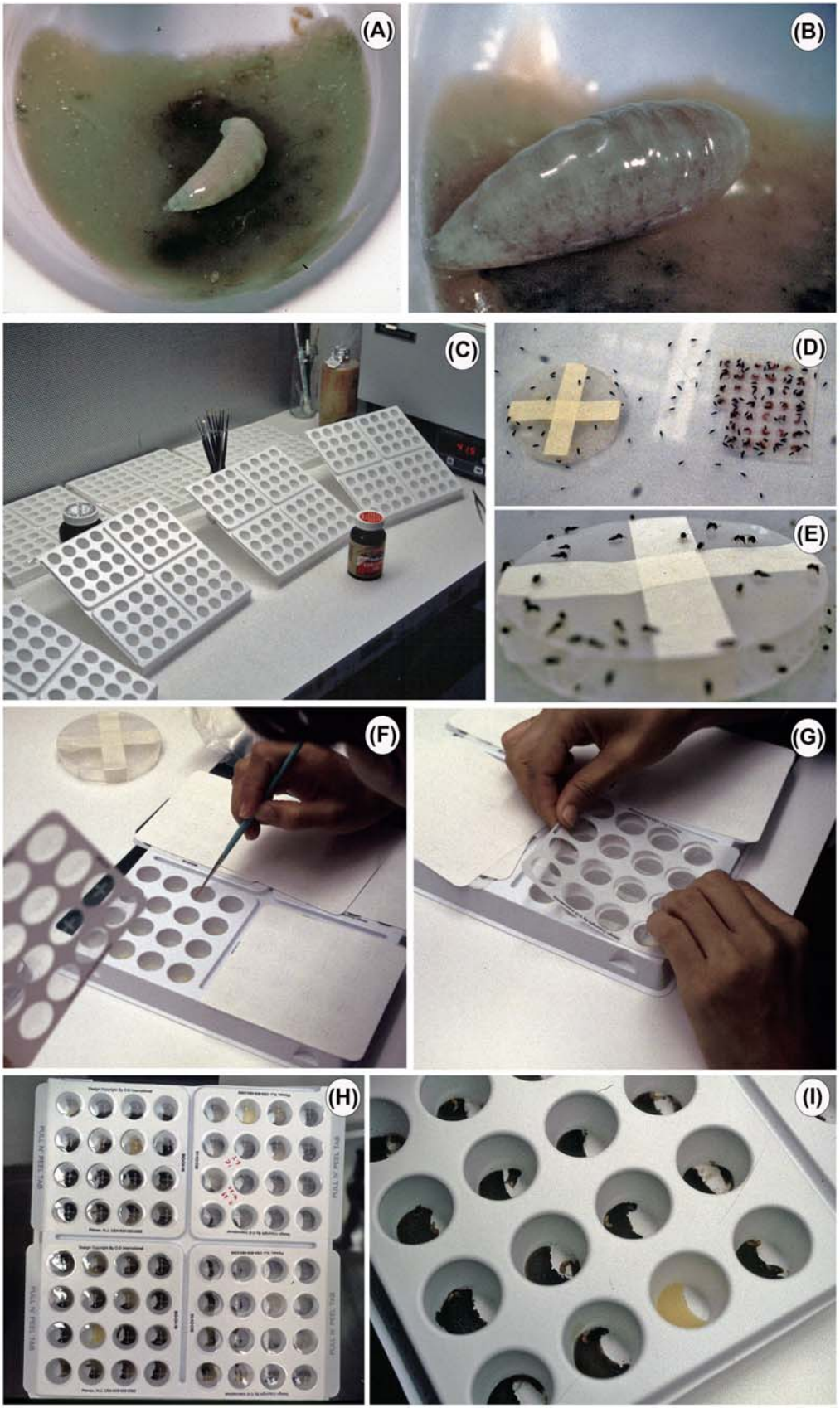

FIGURE 5.16 In vitro rearing of *Catolaccus grandis*. (A) Fourth instar feeding on an artificial diet. (B) Fifth instar feeding on an artificial diet. (C) Trays in 45-degree angle position to be dispensed with artificial diet. (D) Oviposition device next to a sheet of encapsulated boll weevil larvae showing *C. grandis* females ovipositing in both. (E) closeup of oviposition device with ovipositing parasitoid females. (F) parasitoid eggs are placed inside the wells on the side devoid of diet to prevent contamination. (G) After the eggs are positioned, the wells are sealed. (H) tray with developing parasitoids. (I) Open tray with parasitoid pupae.

TABLE 5.9 Composition of the oviposition stimulant for *Catolaccus grandis*.

Ingredient	Percentage
Cholesterol	14.68
Linoleic acid	7.25
Linolenic acid	6.90
Oleic acid	16.35
Palmitic acid	18.20
Palmitoleic acid	3.58
Stearic acid	3.67
Lauryl sulfate[a]	29.36

[a]*Emulsifier.*

5.21 Final remarks and future perspective

The use of parasitoids in biological control was prominent during the 1990s but has not become mainstream despite many confirmed successes, both as classic and augmented biological control agents. The most important reason for the reduced acceptance in mainstream agriculture is the high costs of producing parasitoids and the high requirements for qualified labor. These limitations can be overcome by developing more refined rearing techniques and mechanization. Unfortunately, there has been little emphasis on research directed to insect rearing in the past two decades. The current western agricultural methods based on high input row crops are not compatible with the use of biological control using parasitoids and predators. These agricultural methods, however, are unsustainable and change will be forced on western agriculture in the decades ahead. Biological control by propagation and release of parasitoids and predators will have its place in the future of agriculture. It is important to continue the development of methods to mass-produce these biological control agents because they will play a significant role in sustainable agriculture. It is apparent that biological control by propagation and release of parasitoids and predators has been ahead of its time.

USDA disclaimer

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Chapter 6

Mass-production of arthropods for biological control of weeds: a global perspective

Patrick J. Moran[1], Rosemarie De Clerck-Floate[2], Martin P. Hill[3], S. Raghu[4], Quentin Paynter[5] and John A. Goolsby[6]

[1]*U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS), Invasive Species and Pollinator Health Research Unit, Albany, CA, United States,* [2]*Agriculture and Agri-Food Canada, Lethbridge Research & Development Centre, Lethbridge, AB, Canada,* [3]*Centre for Biological Control, Department of Zoology and Entomology, Rhodes University, Makhanda (Grahamstown), EC, South Africa,* [4]*Commonwealth Science Industry and Research Organisation (CSIRO), Brisbane, QLD, Australia,* [5]*Manaaki Whenua - Landcare Research, Biocontrol and Molecular Ecology Team, Auckland, New Zealand,* [6]*USDA-ARS, Knipling-Bushland Livestock Insects Research Laboratory, Cattle Fever Tick Research Unit, Edinburg, TX, United States*

6.1 Introduction

6.1.1 Theory and rationale for biological control of weeds

Invasive, nonnative weeds reduce availability and quality of water and soil resources, costing at least US$7 billion/year in the US (Duncan and Clark, 2005; Pimentel, 2011) and many threaten biodiversity and natural resources in noncrop areas (DiTomaso, 2000; Pyŝek et al., 2012; Sheley and Smith, 2012). The cost of invasive weeds in noncrop areas globally is at least US$100 billion/year (Pimentel, 2011). Herbicidal and mechanical control are not sufficient for ecosystem-wide management of these weeds (Culliney, 2005; Van Driesche et al., 2010; DiTomaso et al., 2013; Hussner et al., 2017). Classical biological control of invasive weeds (the introduction of nonnative, host-specific natural enemies) is a safe and effective method to manage invasive alien weeds in noncrop areas (Crutwell McFadyen, 1998; Goeden and Andres, 1999; Culliney, 2005; Van Driesche et al., 2010; Clewley et al., 2012; van Wilgen et al., 2013; Suckling and Sforza, 2014; Paynter et al., 2015a,b; Hinz et al., 2019, 2020). Benefit-to-cost ratios range from 8:1 to 300:1 to as high as 7405:1 or more for agents that impact weed survival, growth, reproduction, and population spread (Page and Lacey, 2006; van Wilgen and De Lange, 2011; van Wilgen et al., 2020). Among new agents released since 1991, 70%−80% have established populations, and at least one-third are having sufficient impacts to reduce or eliminate the need for other control methods (Pitcairn, 2018; Schwarzländer et al., 2018).

Through 2016, 493 biological control agents (BCAs) had been released in 1,623 events across 90 countries targeting at least 175 weeds across 48 plant families (Schwarzländer et al., 2018; Hinz et al., 2019, 2020). The most commonly cited risk associated with biological weed control—damage to nontarget plants—is low, with 7.6% of releases leading to some degree of nontarget damage, but less than 1% to sustained damage sufficient to alter nontarget survival or reproduction (Schwarzländer et al., 2018; Hinz et al., 2020). Although many BCAs have established, the risk of establishment failure is substantial, given the up-front investment to discover and characterize new weed BCAs and ensure their safety and efficacy prior to release. Climate and weed genotype matching are used to maximize the likelihood that released BCAs will be preadapted to the areas where they are released (Dhileepan et al., 2006; Goolsby et al., 2006; Zalucki and van Klinken, 2006; Muskett et al., 2020). However, site-specific factors such as predator abundance and release population size (Grevstad, 1999a,b; Fauvergue et al., 2012; Grevstad et al., 2013) can also determine outcomes. Mass-production (henceforth mass-rearing) of BCAs has long been considered a method to increase release population sizes and/or sites and increase the chances of establishment over large geographic areas (Goeden and Andres, 1999; Coulson et al., 2000).

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6.1.2 Scope of chapter

This chapter covers the mass-rearing of arthropods (principally insects) for biological weed control. The focus is mainly on "classical," nonnative BCAs that are imported, tested, and permitted for release in a country or region where a nonnative, invasive weed is causing major environmental and economic damage in noncrop areas. Less commonly, biological control involves the augmentation of native insects that have recruited a nonnative weed as a host. After defining the concept of mass-rearing of weed BCAs, the chapter presents general information on the prevalence of its use, and on critical factors to guide decision-making for the need and feasibility of mass-rearing of weed BCAs. The chapter then presents background information and case studies from five leading countries (in terms of numbers of agents released and weeds targeted) in biological weed control: The United States, Canada, South Africa, Australia, and New Zealand. The chapter concludes with general insights on these studies and proposed future directions for the use of mass-rearing to improve weed biocontrol outcomes.

6.2 Scope of mass-rearing of biological control agents of weeds

6.2.1 Definition of mass-rearing of weed biological control agents

Mass-rearing of BCAs of weeds is defined here as amplification of an insect or mite population under controlled (laboratory or greenhouse) or semi-controlled (field garden or manipulated field site) conditions, to population sizes sufficiently above natural levels to permit distribution of large numbers of arthropods to release sites. "Amplification" of entomophagous beneficial insects has been defined as the rearing of populations that are 10,000 to 1 million times the size that would be attained by females under natural conditions (Chambers, 1977; Leppla, 2014). Rearing programs for weed BCAs are more limited, in most cases, by the need for host-specific, plant-based diets, and by the paucity of large commercial rearing facilities dedicated to rearing them. One of the earliest examples of weed BCA mass-rearing, and one that perhaps achieved the greatest degree of amplification, involved the moth *Cactoblastis cactorum* (Berg) targeting prickly-pear cactus in Australia and South Africa, for which almost 3 billion egg sticks, each containing 10−20 eggs, were produced (Table 6.1). Another example involved the rearing and inundative release of >30 million adults of a native seed-feeding fly *Phytomyza orobanchia* Kalt. for control of parasitic *Orobanche* spp. in tree crops in Russia, Ukraine, Turkmenistan, and Uzbekistan, as well as in Morocco (Klein and Kroschel, 2002). That project was unusual in using a native insect, and in targeting a genus of parasitic weeds in crops.

The rearing of thousands or tens of thousands of individuals in the same laboratory that developed fundamental prerelease knowledge about the produced BCA represents a common "gray area," as a strict definition would involve facilities and personnel dedicated only to mass-rearing (Leppla, 2014). The distribution of agents from nonmanipulated field sites, which is the most common method used to release large numbers of weed BCAs (Blossey et al., 2000; Hansen, 2004; Smith et al., 2009; Van Driesche et al., 2010) does not involve rearing and is not considered further here.

6.2.2 Summary of the extent of use of mass-rearing in weed biological control

The degree of use of BCA mass-rearing in biological weed control varies by country. Authors in the United States have noted the paucity of the use of mass-rearing (Story et al., 1994; Blossey et al., 2000; Hansen, 2004). Of 123 arthropod agents released in the continental United States by 2020 (excludes Hawaii and Alaska) (Winston et al., 2021), 18 were mass-reared, 16 of them nonnative (Table 6.1, Section 6.4.1). Mass-rearing also appears to be infrequent in Canada (Section 6.4.2), where about 12 of 86 total arthropod agents released were mass-reared, and in Australia (Section 6.4.4), where between 10 and 20 of 202 agents (includes pathogens, Schwarzländer et al., 2018) were mass-reared. By contrast, in South Africa, of 87 agents released, 47 have been or are being mass-reared through a government network of facilities (Section 6.4.3), and in New Zealand, 44 of 53 total agents (includes pathogens) have been reared to some degree, although fewer were mass-reared (Section 6.4.5). In most countries, rearing efforts are supported by time-limited weed-specific initiatives funded by government agencies and/or stakeholder groups. Major environmental weed invasions may impact crops, pastures and forest plantations; but mainly impact public goods such as water and soil resources and/or biodiversity (Pimentel, 2011; Pyšek et al., 2012). There is thus little or no economic incentive to produce and sell weed BCAs, compared to the at least US$800 million/per year global industry for the commercial production of about 350 arthropods as BCAs targeting insect pests of crops (van Lenteren et al., 2018), although, according to that paper, about 40 weed BCAs have been produced commercially. Access and benefit-sharing provisions under the Nagoya Protocol and its implementation in signatory countries from which BCAs are sourced (Mason et al., 2018; Smith et al., 2018a,b) must be considered for all weed BCAs, including the likely very small number that can be mass-produced and

TABLE 6.1 Examples of successful mass-rearing of weed biological control agents.

Targeted weed	Insect agent	Host and environment	Number reared[a]	Country (ies) or regions	Reference
Prickly pear cactus (*Opuntia stricta* (Haw.) Haw. (Cactaceae)	*Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae)	Plant-greenhouse and shade house	>2.8 B egg sticks	Australia, Micronesia, Indonesia, South Africa	Dodd (1940), Goeden and Andres (1999), Raghu and Walton (2007)
Hydrilla (*Hydrilla verticillata* (L.f. Royle)) (Hydrocharitaceae)	*Hydrellia pakistanae* Deonier (Diptera: Ephydridae)	Plant-artificial ponds	>28 M immatures	United States	Center et al. (1997), Harms et al. (2009a)
Giant salvinia (*Salvinia molesta* D. Mitch.) (Salviniaceae)	*Cyrtobagous salviniae* Calder and sands (Coleoptera: Curculionidae)	Plant-shade house, outdoor tanks, and artificial ponds	>7 M adults	United States, Australia, South Africa	Flores and Carlson (2006), Harms et al. (2009b), Coetzee et al. (2011), Sullivan et al., (2011), Wahl and Diaz (2020)
Spotted and diffuse knapweed (*Centaurea stoebe* Lam.) and *Centaurea diffusa* Lam.) (Asteraceae)	*Agapeta zoegana* L. (Lepidoptera: Tortricidae) *Cyphocleonus achates* Fahraeus (Coleoptera: Curculionidae)	Plant-caged or uncaged field garden	44,100 adults	United States	Story et al. (1994, 1996)
Scentless chamomile (*Tripleurospermum inodorum* (L.) Sch. Bip.) (Asteraceae)	*Omphalapion hookerorum* (Kirby) (Coleoptera: Brentidae)	Plant-caged field garden	25,000 adults	Canada	McClay and De Clerck-Floate (1999)
Linaria dalmatica L. (Plantaginaceae)	*Mecinus janthiniformis* Toševski and Caldara (Coleoptera: Curculionidae)	Plant-caged field garden	>13,000 adults	Canada	De Clerck-Floate and Harris (2002), Toševski et al. (2018)
Purple loosestrife (*Lythrum salicaria* L.) (Lythraceae)	*Neogalerucella calmariensis* (L.) and *N. pusilla* (Duftschmid) (Coleoptera: Chrysomelidae)	Plant-greenhouse and field garden	>10.6 M adults	United States	Blossey and Hunt (1999), NJDA (2007), MNDNR (2019)
Purple loosestrife	*Hylobius transversovittatus* (Goeze) (Coleoptera: Curculionidae)	Meridic diet with or without plant component	>10,000 adults	United States	Blossey et al. (2000), Tomic-Carruthers et al. (2009)
Mesquite (*Prosopis* spp.) (Fabaceae)	*Evippe* sp. #1 (Lepidoptera: Gelechiidae) and *Prosopidopsylla flava* (Burckhardt) (Hemiptera: Psyllidae)	Plant-greenhouse	62,000 larvae and 183,000 adults	Australia	van Klinken et al. (2003)
Mile-a-minute weed, *Persicaria perfoliata* (L.) H. Gross) (Polygonaceae)	*Rhinoncomimus latipes* Korotyaev (Coleoptera: Curculionidae)	Plant-greenhouse	>535,000 adults	United States	NJDA (2007), Hough-Goldstein et al. (2008, 2009), Lake et al. (2014)
Cat's claw creeper (*Dolichandra unguis-cati* (Linné) L. G. Lohmann) (Bignoniaceae)	*Carvalhotingis visenda* (Drake & Hambleton) (Hemiptera: Tingidae)	Plant-greenhouse and field garden	>0.5 M adults and nymphs	Australia	Dhileepan et al. (2010)

(*Continued*)

TABLE 6.1 (Continued)

Targeted weed	Insect agent	Host and environment	Number reared[a]	Country (ies) or regions	Reference
Tropical soda apple (*Solanum viarum* Dunal) (Solanaceae)	*Gratiana boliviana* Spaeth (Coleoptera: Chrysomelidae)	Plant-greenhouse	>251,000 adults	United States	Diaz et al., 2014
Air potato (*Dioscorea bulbifera* L.) (Dioscoreaceae)	*Liliocerис cheni* Gressitt and Kimoto (Coleoptera: Chrysomelidae)	Plant-laboratory and shade house	>812,000 adults and larvae	United States	Overholt et al. (2016), FDACS (2020)
Arundo (*Arundo donax* L.) (Poaceae)	*Rhizaspidiotus donacis* Leonardi (Hemiptera: Diaspididae)	Plant-greenhouse	>4500 adult females	United States	Villarreal et al. (2016)
Brazilian peppertree (*Schinus terebinthifolius* Raddi (Anacardiaceae)	*Pseudophilothrips ichini* (Hood) (Thysanoptera: Phlaeothripidae)	Plant-greenhouse	>1 M adults	United States	Halbritter et al. (2021)
Arundo	*Tetramesa romana* Walker (Hymenoptera: Eurytomidae)	Plant-greenhouse	1.2 M adults	United States	Section 6.4.1.2
Houndstongue (*Cynoglossum officinale* L.) (Boraginaceae)	*Mogulones crucifer* (Pallas) (Coleoptera: Curculionidae)	Plant-field garden	>162,000 adults	Canada	Section 6.4.2.2
Water hyacinth (*Pontederia (Eicchornia) crassipes* (Mart.) Solms.) (Pontederiaceae)	*Megamelus scutellaris* Berg.	Plant-greenhouse and outdoor field tanks	>4.5 M adults and nymphs	South Africa, United States	Section 6.4.3.2, Tipping et al. (2014), Moran et al. (2016), Freedman and Harms (2017), Goode et al. (2021), P. Moran, unpubl.
Parkinsonia (*Parkinsonia aculeata* L.) (Fabaceae)	*Eueupithecia cisplatensis* Prout and *Eueupithecia vollonoides* Hausmann (Lepidoptera: Geometridae)	Plant-greenhouse and manipulated field sites	>1.38 M larvae and pupae	Australia	Section 6.4.4.2
Japanese honeysuckle (*Lonicera japonica* (Thunb.)) (Caprifoliaceae)	*Limenitis glorifica* Fruhstorfer (Lepidoptera: Nymphalidae)	Plant-greenhouse and shade house	57,185 adults	New Zealand	Section 6.4.5.2
Tradescantia or spiderwort (*Tradescantia fluminensis* Vell.) (Commelinaceae)	*Neolema ogloblini* (Monrós) (Coleoptera: Chrysomelidae)	Plant-greenhouse	25,050 adults	New Zealand	Section 6.4.5.3

[a]*M = Million, B = Billion.*
Source*: Table adapted and expanded from the first edition of this book.*

sold for profit. However, in countries where government agencies develop weed BCAs, their commercialization is often prohibited. The main factors that limit the use of mass-rearing of weed BCAs are costs, logistics (see country sections of this chapter), and insect biological factors, such as univoltinism (Story et al., 1994, 1996).

6.2.3 Benefits of mass-rearing in biological weed control

A long-standing debate in biological weed control concerns the need to release large numbers of BCAs at one or a few sites (inundative: may apply to nonnative or native agents), or small numbers at many sites (inoculative) (Goeden and Andres, 1999; Grevstad, 1999a,b; Fauvergue et al., 2012; Grevstad et al., 2013). Either approach may create an incentive for mass-rearing. Releasing small numbers of reproductive individuals at a large number of sites may increase the likelihood of establishment across the range of introduction (Grevstad et al., 2013; Lake et al., 2018). Mass-rearing may thus help overcome isolation effects, generalist predators and climate extremes at each site (Goeden and Louda, 1976; Crawley, 1986; Memmott et al., 1998; Grevstad et al., 1999a,b; Keane and Crawley, 2002) and increase the rate of establishment across countries or regions. Modern biological control practices include identifying the most vulnerable stage in the weed's life history (Blossey et al., 2018). Mass-rearing may be motivated by the need to release large numbers of BCAs at the most vulnerable stage, for example early in the field season to damage young plants, when agent populations might otherwise be limited by seasonal climate (Frick and Garcia, 1975).

6.3 Critical factors in the design and use of mass-rearing protocols in biological weed control

6.3.1 Decision-making regarding the need for mass-rearing

Due to the significant costs, a decision to mass-rear one or more BCAs rests largely on the real and perceived economic damage being caused by the targeted weed. Modern biological control projects include cost-benefit analyses and risk analyses to help guide these decisions (van Wilgen et al., 2020). Mass-rearing may involve only agents that offer the highest expected benefit-to-cost ratio. However, actual (as opposed to predicted) benefits of release are unknown at the start of the rearing project, unless the agent has been released elsewhere; thus, realized damage caused by the weed is likely to drive decisions. Other factors include the ability of the agent to self-disperse and the speed with which country- or region-wide establishment is desired. The goals of mass-rearing must be aligned with the larger goals of the biological control project, which should consider the population dynamics of both the weed and BCA (Schaffner et al., 2020); these in turn must function in the context of an adaptive, integrated weed management plan.

6.3.2 Decision-making regarding the feasibility of mass-rearing

"Ease of culturing" is often a factor in determining whether to pursue studies of the host range, biology and predicted efficacy of a candidate weed BCA (Goeden and Andres, 1999; Sheppard and Raghu, 2005). Small-scale rearing in native range and quarantine laboratories is needed to determine the candidate's life cycle and perform host range and laboratory efficacy studies (Price, 1991; Moran and Goolsby, 2009, 2010). Mass-rearing requires not only scale-up of these procedures but also their modification for practical environments, including facilities that are not research laboratories. The processes by which plant-feeding insects select host substrates for oviposition and feeding are complex. A myriad of abiotic factors, including light, temperature, humidity and the presence of nonhost stimuli, influence substrate selection (Bernays and Chapman, 1994; Chapman, 2003; Müller-Schärer and Schaffner, 2008). The physiological and biochemical state of the host plant plays a critical role in determining plant-feeding insect survival and reproduction (Rohrfritsch, 1992; Waring and Cobb, 1992; Price, 2000; Kerchev et al., 2012; Moran and Goolsby, 2014; Moran, 2015; Portman et al., 2021). Feasibility assessment and preparation for mass-rearing, therefore, involves experimental determination of key factors that govern the insect life cycle, building biological knowledge of the agent typically beyond that elucidated in quarantine.

As can be seen from the case studies below, successful mass-rearing programs for weed BCAs always require involvement and support from multiple government agencies, academic or extension faculty, and stakeholder groups, and their availability must be considered in determining feasibility. In some cases, in the United States (MNDNR, 2019), South Africa (Weaver et al., 2017) and elsewhere, primary and secondary schools, as well as private citizen groups with interests in natural resource protection, have been critical participants.

6.3.3 Critical factors in the design of mass-rearing protocols

6.3.3.1 Production of host substrates

In almost all cases, mass-production of the targeted weed plant host is required for mass-rearing of weed BCAs. In a few cases, an artificial diet has been developed (Blossey et al., 2000; Wheeler and Zahniser, 2001), but the use of artificial diets is limited by the need for multiple stimuli provided only by the host, by the need for time-consuming studies to determine diet requirements, and by logistical and resource constraints. Mass-production of a weed as a greenhouse or field crop carries its own challenges, including seed germination (or propagule sprouting for asexually-reproducing species), maintenance of vigorous, healthy, pest-free vegetative growth (Price, 2000) and, for BCAs that attack flowers or seeds, induction of flowering and maintenance for the full weed life cycle. Weed growing conditions must often be adjusted and optimized.

6.3.3.2 Knowledge of agent population biology and plant-insect ecology

Modern biological control systems require the use of genetically-identified BCA material from a known geographic source, due to concerns about cryptic species (Smith et al., 2018a,b) and variation in host range between distant populations of the same BCA species (Paynter et al., 2008). Agent damage may induce changes in host plant quality (Wheeler and Schaffner, 2013; Barrett et al., 2021), requiring transfers or precluding rearing on regrowth. Even if weed host material can be optimized for BCA development and reproduction, development is dependent on temperature, as described in degree-day equations (Zalom et al.,1983). Degree-day studies determine a "threshold temperature" below which the BCA cannot develop and can be used to infer an "optimal" temperature that maximizes population growth as well as the range of temperatures at which the BCA can reproduce. Degree-day information is not typically developed during quarantine rearing. If determined shortly after the first release (May and Coetzee, 2013), it can be useful for mass-rearing, but more often it is generated later, to help interpret limitations on establishment or dispersal (Willden and Evans, 2018). Mass-rearing protocols may have to rest in part on degree-day or more recent nonlinear models (Ji et al., 2021; Santos and Marchioro, 2021) to determine optimal rearing temperature and lower and upper thresholds based on information for related species, possibly crop pests. Mass-rearing systems can be thought of as artificial, *n*-dimensional ecological niches, in which temperature, humidity, lighting, gas exchange and diet quality interact with each other to influence insect biology (Carson, 2021). The influence of environmental parameters other than temperature on the BCA that were not determined in quarantine must be elucidated to carry out scientifically-informed mass-rearing (Blossey and Hunt, 1999; Blossey et al., 2000; Harms et al., 2009a,b; Freedman and Harms, 2017). These include moisture, light intensity and daylength requirements to maximize adult emergence, survival, mating, oviposition, immature development, pupation as applicable, and overwintering survival.

6.3.4 Implementation of mass-rearing

6.3.4.1 Monitoring of output level and efficiency

Once resources and personnel are in place, either at a small number of large facilities or distributed across a wide range of technical and nontechnical rearing partners, it is critical to continuously monitor the BCAs output (Moran et al., 2014); see case studies in Section 6.4. Productivity should be measured in terms of output:input ratios (based on input females) to track efficiency. Morales-Ramos et al. (2014), Portilla et al. (2014), Leppla (2014) and chapters in this book cover methods to track the success of rearing method development and output BCA quality in terms of life tables. Weed BCAs mass-reared under controlled (lab, greenhouse) or semi-controlled (shade house, field garden, manipulated field site) conditions may experience inbreeding or adapt in ways that reduce their ability to establish under open field conditions (Hopper et al., 1993; Blossey and Hunt, 1999). Quality control procedures should be implemented, including periodic tests of newly-reared agents in cages in the field, with comparison to cages populated by established populations. When centralized facilities are used, the cost of mass-rearing per arthropod individual should be tracked and remain steady over time or decrease as a result of rearing process improvements.

6.3.4.2 Evaluation of success of mass-rearing

Success in mass-rearing can be evaluated in terms of total output (Table 6.1) but is more usefully evaluated in terms of the number of individuals released per site and across all sites, with subsequent consideration of site establishment rates (Hinz et al., 2020; see case studies, Section 6.4). Comparisons of mass-reared versus other released agents are not likely to show a benefit in terms of establishment, as the global establishment rate for all agents is between 60% and 85%

(Schwarzländer et al., 2018) higher than the global percentage of agents that were mass-reared (ca. 25% based on the numbers provided in Section 6.2.2). Success could be evaluated in terms of percentage of sites established (Grevstad et al., 2013), or establishment at the highest-priority sites, such as upstream "nursery" sites from which the BCAs can easily-self-disperse or be collected and dispersed by people. Evaluation of success in terms of impact on the weed is beyond the scope of mass-rearing but is likely to be included in assessments. Success in biological weed control was traditionally defined as the widespread establishment of one or more BCAs and easily-observable damage to the targeted weed ("'before/after" field site pictures) (Schaffner et al., 2020), but in modern systems success is evaluated in terms of population-level effects on weed survival, reproduction, dispersal and range expansion, with evidence of reduction in economic and environmental damage caused by the weed (Schwarzländer et al., 2018; Hinz et al., 2020). Success in mass-rearing is often also evaluated in terms of outreach and technology transfer to natural resource managers, landowners, and other stakeholders (Diaz et al., 2014; see case studies from South Africa and Australia, Section 6.4).

6.4 Case studies on mass-rearing in biological weed control

6.4.1 United States

6.4.1.1 History of use

The information here considers the continental United States (CONUS); for information on biological weed control in Hawai'i, see Markin et al. (1992); Conant et al. (2013), and Schwarzländer et al. (2018). The first biological control project in CONUS (and all of North America), using nonnative BCAs involved St. Johnswort or Klamath weed (*Hypericum perforatum* L.) (Hypericaceae) and two chrysomelid beetles (*Chrysolina hyperici* (Forster) and *C. quadrigeminata* Suffrian). Starting from 10,000−15,000 (mainly) adults that were field-collected from successful Australian releases and released in California in 1945−46 (Holloway, 1948), in 1950 over 3 M adults of *C. geminata* were collected from US sites and redistributed (Holloway and Huffaker, 1951). This project, one of the most successful in US history (Pitcairn, 2018), thus did not involve mass-rearing, as was the case for tansy ragwort, *Jacobaea vulgaris* Gaertn. (Asteraceae) targeted with a leaf-feeding moth *Tyria jacobaeae* (L.) (Lepidiotera: Erebidae) from France and a root and rosette-feeding flea beetle (*Longitarsus jacobaeae* Waterhouse) (Coleoptera: Chrysomelidae) from Italy and Switzerland (Turner and McEvoy, 1995; Coombs et al., 2004). Another early project involved two weevils (*Microlinus* spp.) feeding on stems and seeds of puncturevine *Tribulus terrestris* L. (Zygophyllaceae) (Andres and Goeden, 1995). Examples of other successful programs in the United States that did not initially involve mass-rearing of weed BCAs include those targeting alligator weed (Coulson, 1977), waterhyacinth (Center et al., 2002), leafy spurge (Hansen et al., 1997), and tamarisk (Knutson et al., 2019), except in far northern areas (Dr. Sharlene Sing, US Forest Service, Bozeman, MT United States, personal communication).

Overall, in CONUS 123 weed BCAs have been released against 58 weeds since 1945, and 95 agents are established against at least one weed (Winston et al., 2021), with six additional released agents being too early to determine. Work from the 1940s through the 1960s, conducted mainly by the US Department of Agriculture-Agricultural Research Service (USDA-ARS) focused on the overseas collection, quarantine rearing, and release of small numbers of agent individuals, followed by technology transfer to collaborators for redistribution (Coulson, 1977). The earliest uses of mass-rearing in weed biological control in the US occurred in the 1970s, but did not involve nonnative agents. In a rare application to crop environments, a meridic diet with dried powder plant components was used to rear a native moth, *Bactra verutana* Zeller (Lepidoptera: Tortricidae) targeting purple nutsedge, *Cyperus rotundus* L. (Cyperaceae) in cotton (Garcia and Frick, 1975; Frick and Garcia, 1975; Frick and Chandler, 1978). A total of 10,000 larvae of a native bud-feeding moth in the Pterophoridae were reared on plant material of a peri-agricultural weed, native hedge bindweed (*Calystegia sepium* (L.) R. Br.) (Parrella and Kok, 1979). The use of mass-rearing of nonnative BCAs increased in the 1990s for agents on hydrilla, giant salvinia, knapweeds, and purple loosestrife, and its use has since continued to expand (Table 6.1).

There is no coordinated national framework or agency that conducts mass-rearing of weed BCAs in the United States. The US Department of Agriculture-Agricultural Research Service (USDA-ARS) develops small-scale rearing technology for candidate weed BCAs (Portman et al., 2021), with scale-up for initial field releases (Tipping et al., 2014), and then transfers technology to other Federal or state government agencies or university cooperators for mass-rearing, if funding is available. The USDA-ARS Invasive Plant Research Laboratory in Ft. Lauderdale, Florida has cooperated with Federal-state interagency groups, state agencies, and university research and extension faculty to mass-rear agents for release on melaleuca (Center et al., 2000), water hyacinth (Tipping et al., 2014), air potato

(Overholt et al., 2016), Old World climbing fern (Smith et al., 2014) and most recently Brazilian peppertree (Halbritter et al., 2021) (Table 6.1). The USDA-Animal and Plant Health Inspection Service (USDA-APHIS) mass-reared and released the giant Salvinia weevil (Flores and Carlson, 2006; Table 6.1). The US Army Corps of Engineers, Aquatic Plant Control Research Program in Vicksburg, Mississippi has performed similar activities targeting aquatic weeds, including hydrilla, giant salvinia, and water hyacinth (Harms et al., 2009a,b; Freedman and Harms, 2017) (Table 6.1). At least three US states have government facilities dedicated in part to mass-rearing of weed BCAs: Florida (Lake et al., 2017), New Jersey (NJDA, 2007), and Colorado (CDA, 2016).

University researchers have also developed and implemented mass-rearing and/or coordinated technology transfer. Three established BCAs of purple loosestrife were mass-reared based on procedures developed at Cornell University (Ithaca, New York), including two leaf-feeding beetles and a root-feeding weevil (Table 6.1) (Grevstad, 2006). Rearing of the leaf beetles in artificial ponds led to five-fold more production than that achieved in a greenhouse (Blossey and Hunt, 1999). The root weevil was reared on a meridic diet with plant components (Blossey et al., 2000). A program targeting mile-a-minute weed with a chrysomelid beetle was initiated with technology developed at the University of Delaware (Newark, Delaware) (Hough-Goldstein et al., 2009), and then implemented at a rearing facility operated by the New Jersey Department of Agriculture (Table 6.1). Researchers at the University of Florida led a successful rearing and implementation program for a chrysomelid beetle targeting tropical soda apple across the southeastern United States (Table 6.1) (Diaz et al., 2014).

Almost all mass-rearing of weed BCAs in the United States has been conducted on host plants in greenhouses, shade houses, outdoor tanks or field gardens. Rearing of *H. transversovittatus* represents the only production-scale use of an artificial diet for a nonnative weed BCA in the United States, also a rare success worldwide. The diet was later refined to remove the host plant root component (Tomic-Carruthers, 2009) (Table 6.1) and its use has continued (CDA, 2016). Meridic diets were developed by USDA-ARS for the melaleuca weevil *Oxyops vitiosa* Pascoe (Wheeler and Zahniser, 2001) and by USDA-APHIS for the knapweed root weevil *Cyphocleonus achates* (Fahraeus) (Goodman et al., 2006; Tomic-Carruthers, 2009), but were not used in mass-production.

6.4.1.2 Case study: a shoot tip-galling wasp on arundo

The shoot tip-galling wasp *Tetramesa romana* Walker (Hymenoptera: Eurytomidae), which feeds on the giant grass known as arundo, giant reed or carrizo cane (*Arundo donax* L.) is the first, and thus far only gall-making member of this wasp family released for biological weed control, and one of only two released members of this family; the other, *Eurytoma attiva* Burks, is a seed-feeder on black sage, *Cordia curassavica* (Jacq.) (Roem. & Schult.) (Simmonds, 1980). A stem-boring congener, *Tetramesa phragmitis* (Erdos) is present in adventive populations on common reed (*Phragmites australis* (Cav.) Trin ex Steud subsp. *australis*) in the United States (Tewksbury et al., 2002). Adult female *T. romana* wasps (Fig. 6.1A) emerge with fully developed eggs and reproduce parthenogenetically, depositing eggs singly into shoot tips of arundo (Fig. 6.1B), (Goolsby and Moran, 2009; Moran and Goolsby, 2010). Eggs initiate gall formation and larvae complete three instars, pupate in galls (Fig. 6.1C), chew exit holes and emerge as adults, completing the life cycle in 35−40 days at a constant 27°C or 32°C (Moran and Goolsby, 2009; Moran et al., 2014). Arundo wasp

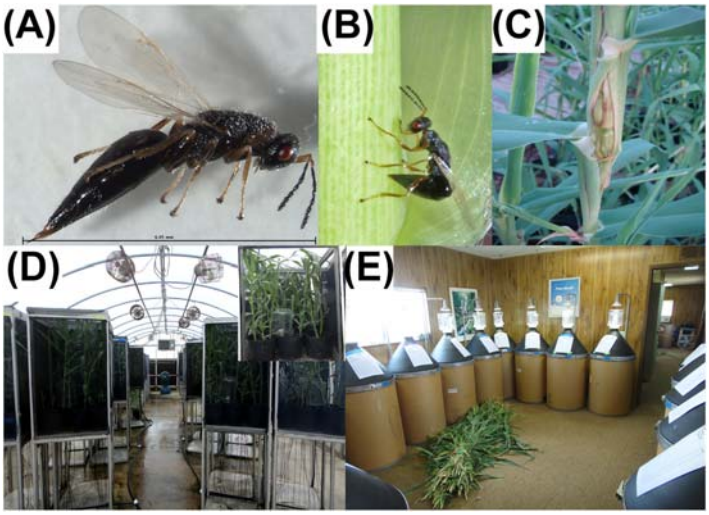

FIGURE 6.1 Mass-rearing of the arundo wasp in greenhouses in the United States (A) Adult wasp. (B) Female ovipositing into arundo shoot tip. (B) Large gallon main shoot. (D) Oviposition greenhouse and cages. Inset shows a cage filled with nine potted arundo shoots. (E) Wasp emergence room with shoots on floor awaiting placement in cardboard barrels with black conical tops and lights to attract wasps for collection into plastic jars. Photo credit: USDA-ARS. (A), (D) and (E) are from the first edition of this book. (For a color version of this figure, the reader is referred to the online version of this book).

galls are monothalamous (single-chambered) (Askew, 1984; Rohrfritsch, 1992) and closely packed at shoot tips (Fig. 6.1C). A mass-rearing program was developed to release wasps from Mediterranean Spain, the known area of origin of invasive arundo populations (Tarin et al., 2013).

6.4.1.2.1 Key challenges in designing a mass-rearing program

Arundo wasp females deposit eggs at shoot tip nodes (Moran et al., 2014) and in the field they induce galls on both main and lateral shoots, with the latter being more abundant (Goolsby et al., 2014; Marshall et al., 2018). Oviposition and/or the presence of eggs is sufficient to induce gall formation (Bronner, 1992), with larval feeding required for complete gall maturation (Silva and Shorthouse, 2006). The objective of the mass-rearing program was to produce many large galls on caged, potted main shoots (Fig. 6.1D). Behavioral observations of females and galling efficiency studies had shown that bright sunlight was needed for oviposition and that both high relative humidity (80%) and adult honey provisioning in cages prolonged adult lifespan (Moran et al., 2014). Galls of other insects contain tissues with high protein, amino acid, and low molecular weight carbohydrate content (Bronner, 1992; Rohrfritsch, 1992; Raman et al., 2005) and are potent nutrient sinks (Rohrfritsch, 1992; Florentine et al., 2005; Moseley et al., 2009; Marini-Filho and Fernandes, 2012; Nabity et al., 2013; Dsouza and Ravishankar, 2014; Oliveira et al., 2017; Hirano et al., 2020; Poudel et al., 2020). Gall-formers are thus expected to select vigorous tissues for oviposition (Price, 1991, 2000; Huberty and Denno, 2004). Survival, development, reproduction, and/or densities of galling insects are generally reduced on water-stressed plants (Waring and Cobb, 1992; Horner and Abrahamson, 1999; Björkman, 2000; Huberty and Denno, 2004; Raman et al., 2005; Oliveira et al., 2013; Dardeau et al., 2015; Price and Hunter, 2015), and this trend includes stem-galling biological control insects (Harris and Shorthouse, 1996; Hinz and Müller-Scharer, 2000; Dhileepan, 2003, 2004; Portman et al., 2021). Observations and experimental studies demonstrated positive effects of nitrogen addition (faster time from oviposition to adult emergence, Moran and Goolsby, 2014) and negative effects of water deficit stress (delayed adult emergence, Moran, 2015) on the arundo wasp. Potted arundo plants were fertilized with urea and watered abundantly in the mass-rearing system. Wasp accessions were collected from areas of the native range where arundo was most similar genetically to invasive southern Texas populations (Tarin et al., 2013). Five wasp accessions, including three from the southern and eastern Mediterranean coast of Spain (Las Cañas, Granada, and Caloma), one from southwestern France along the Mediterranean coast (Ceret), and an adventive Texas accession found near Laredo, Texas (Racelis et al., 2009) were mass-reared. Efforts to develop an artificial diet for *T. romana* were not successful (Moran et al., 2014).

6.4.1.2.2 Summary of the mass-rearing protocol

Rearing was conducted at the USDA-APHIS Plant Pest Diagnostic and Management Laboratory, Edinburg, Texas. Wasps were initially emerged from European-collected or adventive Texas field galls in Plexiglas boxes in a growth chamber at 25°C−30°C and 14:10 fluorescent light:dark cycle. Cages were misted once per day with a handheld pump sprayer and provisioned with a ca. 0.5 cm-wide streak of honey. Males are sterile, and so the female:male collection ratio was over 9:1 (Moran and Goolsby, 2009; Goolsby et al., 2014). Plants were reared from rhizomes in 20-cm pots of soil (Sunshine Mix #1, Sun-Gro Horticulture, Agawam, MA, United States) supplemented with 10 g urea, in a greenhouse maintained between 25°C and 35°C with daily abundant watering with emitters or overhead spray irrigation. Plants were 0.5−0.8 m tall when used for wasp infestations, and 200−300 plants were produced per week. Between 6−8 plants (6−32 shoots) and 70−100 wasps were placed into each oviposition cage (Fig. 6.1D), 6.1 m tall by 0.8 m wide by 0.8 m deep, elevated ca 0.8 m off the ground for increased sunlight and ease of access (known as the "Sands design," after Dr. Don P. A. Sands, formerly of CSIRO, Australia; Moran et al., 2014). The number of cages infested per wasp accession per week was highest (6−8) between January and April, and in October, for spring and fall field releases, respectively. Black nylon or silk (mesh size 20 or about 0.2 mm) was stretched over the aluminum cage frame to confine wasps. The greenhouse roof and cage material reduced light levels by over 50%. Conditions were suitable for rearing under ambient light between March and November. Sodium halide lamps (1000 W) were used during the winter months between 1700 and 2000 hours. After 7 days (by which time adult wasps had died, Moran and Goolsby, 2009) plants were removed, fertilized again with 10 g urea, and maintained in a separate greenhouse under ambient light at 30°C−35°C, or in a shade house under ambient conditions in the summer to reduce cooling costs, and in winter to slow wasp development rate, when production was maintained for colonies and not field releases. Plants received the third dose of urea 28 days after removal from oviposition cages.

Except in winter, shoot bases and tips were cut 35 days after plant removal from cages, cut ends sealed with paraffin wax, and all shoots placed inside 208 L cardboard barrels with 60 degree angled-conical tops threaded on top to hold an

inverted 3.8 L plastic jar (Fig. 6.1E). Attached to the top of each barrel was an LED light (18 W, 1000 lumens). The adult emergence room was maintained at ca. 25°C−30°C. Wasps were collected every 1−2 days with handheld vacuum aspirators into 15 mL vials. Each barrel contained one week (5−8 oviposition cages) of shoots for one wasp accession.

6.4.1.2.3 Summary of output of mass-rearing and cost

The rearing program ran for 4 years, from 2009 to 2012. A typical week of cages (i.e., one emergence barrel) produced progeny of 500 to 3000 individuals for each wasp accession. Production across all accessions peaked at about 3000 wasps per day in the spring and early summer (Moran et al., 2014). A total of 1.2 million wasps were produced and released aerially (Racelis et al., 2010) or on the ground at 20 sites along the Rio Grande in Texas between Brownsville and Del Rio (Goolsby et al., 2014) at a cost of about US$0.25/wasp. Additional wasps were sent to three sites in California, United States, and to several sites in Mexico, or were used to initiate wasp populations on farm-raised Arundo in Texas for further releases.

6.4.1.2.4 Impact of mass-rearing on agent establishment and efficacy

The arundo wasp established populations at 15 sites covering 500 river miles of the Rio Grande by 2013 and the adventive Texas population dominated field populations three years after release. (Goolsby et al., 2014). The arundo wasp has reduced live arundo biomass by up to 44%, fostering two-fold increases in the abundance of other plants at sites in the Lower Rio Grande Basin of Texas (Goolsby et al., 2016; Moran et al., 2017; Goolsby and Moran, 2019). Field-collected and farm-raised wasps from south Texas were released in northern California, and the wasp has established populations there (Moran, 2021). The wasp has been released in New Zealand (Winston et al., 2021) and is being considered for release in Australia. It is adventive in South Africa (Canavan et al., 2019) and southern California (Dudley et al., 2008).

6.4.2 Canada

6.4.2.1 History of use

Canada's classical weed biological control program has been active in the introduction of arthropod agents for 70 years, with notable successes particularly in western regions of the country (i.e., montane grasslands of southern British Columbia, and the Prairies of southern Alberta, Saskatchewan and Manitoba) (De Clerck-Floate and Cárcamo, 2011; Vankosky et al., 2017). A total of 86 foreign arthropod species (85 insects + one mite) have been deliberately released against 34 invasive alien plant (IAP) species since the national program's inception (Winston et al., 2021). Near 70% (59/86) are currently established, with the majority of these from the insect Orders, Coleoptera (31 species; ∼ 53%), Diptera (13 species; 22%), and Lepidoptera (11 species; ∼19%). These results closely align with global trends in weed biological control, including the predominant use and success of Coleopteran species in achieving establishment and weed control (Schwarzländer et al., 2018). Canada's top eight performing weed BCAs to date are all Coleoptera, including five weevils (Curculionidae). Besides their proven efficacy, beetles have a reputation within the Canadian program for being hardy in the harsh winter climates and also capable of surviving stresses imposed during field collection, rearing, long-distance transport, and release.

There are no federally-dedicated personnel and laboratory facilities for large-scale weed BCA propagation. National support for weed biological control implementation in Canada is instead research-based, with a focus on getting federal government approval for the importation of new BCAs and on developing release strategies to optimize their establishment success. Federal weed biological control researchers typically establish BCA colonies at their government facilities and rear them in sufficient numbers for experimental releases. The operational side of weed biological control in Canada, including the mass-rearing and distribution of agents, is typically implemented at the regional level by stakeholders with land management responsibilities/interests, including local weed control (e.g., provincial and municipal governments, producer and environmental organizations, transportation and energy companies, and First Nations groups). Researchers provide verified, pure colonies to stakeholders for their own initial field releases and/or for set-up of short-term, typically low-input field garden mass-rearing to boost agent numbers for subsequent regional-scale distribution.

Temporarily boosting agent numbers early in a project has greatly helped get several effective agents more quickly and widely established while also engaging stakeholders. One example involved a campaign in British Columbia to distribute and establish the stem-boring weevil *Mecinus janthiniformis* Toševski and Caldara (Coleoptera: Curculionidae) targeting Dalmatian toadflax (*Linaria dalmatica* L.) (Plantaginaceae). The few weevils first received in 1991−92 were

used for initial field releases, but also for mass-rearing within caged garden plots of grown and tended (i.e., trimmed and watered) plants (De Clerck-Floate and Harris, 2002; Toševski et al., 2018). In 1994, near 6000 weevils were harvested from propagation plots, allowing for 19 releases across British Columbia (De Clerck-Floate and Harris, 2002), and many of these established and quickly became key unmanaged field nursery sites for *M. janthiniformis* redistributions (De Clerck-Floate and Turner, 2013). By 2000, the propagation plots were dismantled, but they had produced +7000 more weevils for early distribution (Table 6.1). Similarly, short-term outdoor mass-rearing was used by stakeholder groups in Manitoba and Saskatchewan to temporarily boost early populations of the purple loosestrife (*Lythrum salicaria* L.) (Lythraceae) biocontrol beetles, *Neogalerucella calmariensis* (L.) and *N. pusilla* (Duftschmid) (Coleoptera: Chrysomelidae) for initial distributions at nursery sites (Lindgren et al., 2002) which, after successful establishment, were designated for redistribution. Also, a seed-feeding beetle *Omphalapion hookerorum* (Kirby) (Coleoptera: Brentidae) [previously *Omphalapion hookeri* (Kirby)] was temporarily mass-reared in garden cages for initial successful field establishment in Prairie provinces on scentless chamomile (McClay and De Clerck-Floate, 1999; Table 6.1). Currently, there is no active "agent boosting" campaigns in Canada, except for a new agent, *Hypena opulenta* (Christoph) (Lepidoptera: Erebidae) for dog-strangling vine (*Vincetoxicum rossicum* (Kleopow) Barbar.) (Asclepiadaceae) in Ontario. Laboratory propagation of the moth on living host plant material is being conducted seasonally by a private company, with support from local land managers (R. Bourchier, Agriculture and Agri-Food Canada, personal communication, February 22, 2021).

6.4.2.2 Case study: a root weevil on houndstongue

The European-sourced root weevil, *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) (formerly *Mogulones cruciger* Herbst), was approved for release in Canada in 1997 for biological control of the invasive rangeland weed houndstongue (*Cynoglossum officinale* L.) (Boraginaceae) (De Clerck-Floate, 2013). This BCA proved to be highly effective in establishing, dispersing, and controlling houndstongue in British Columbia (De Clerck-Floate and Wikeem, 2009), thereby creating a large demand for the weevil among regional stakeholders. Adult feeding by *M. crucifer* in spring (Fig. 6.2A), and subsequent root-mining by larvae (Fig. 6.2B), were shown to cause substantial mortality (Fig. 6.2C), especially when *M. crucifer* numbers were at outbreak levels (Catton et al., 2016). Individual females of this univoltine weevil are long-lived and can produce 250 + eggs, which are mostly laid in April−May into the base of houndstongue leaf petioles (Schwarzländer, 1997). Larvae mine into the root, with large numbers sometimes completing larval

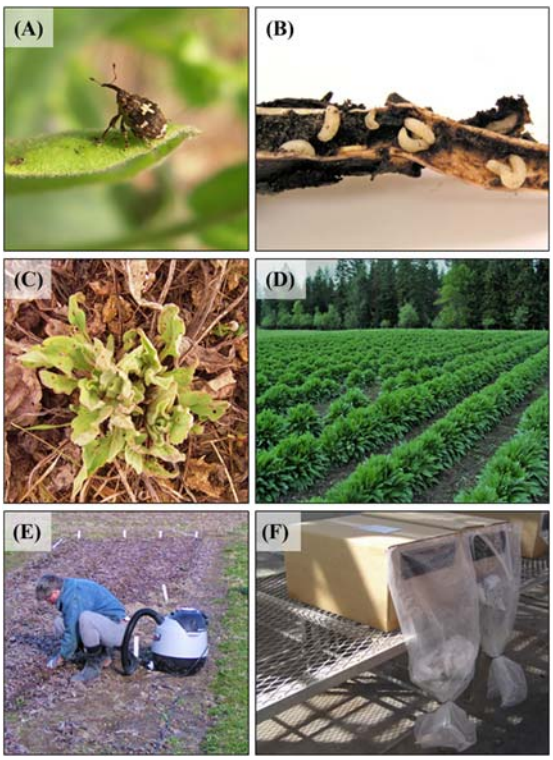

FIGURE 6.2 Mass-rearing of the weevil *Mogulones crucifer* in cultivated plots in Canada. (A) Adult feeding in spring on leaf. (B) Larvae mining roots in summer. (C) Larval and adult feeding causes distortion and death of houndstongue. (D) Cultivation of houndstongue for rearing. (E) Use of wet-dry vacuum to collect adult beetles emerging in spring from "trap" plants. (F) Emergence box to separate weevils from vacuumed debris. Photo credit: R. DeClerck-Floate, Agriculture and Agri-Food Canada. (For a color version of this figure, the reader is referred to the online version of this book).

development within a single houndstongue taproot (Van Hezewijk et al., 2008). Third instar larvae exit roots in July to pupate in the soil, and adults emerge in late summer to feed on houndstongue rosette leaves before overwintering in leaf litter. Of relevance to biological control implementation, *M. crucifer* adults are only noticeably active on houndstongue during spring mating and oviposition, but due to their cryptic brown and black coloration, and a habit of falling to the ground when disturbed, they are difficult to collect.

6.4.2.2.1 Key challenges in designing the mass-rearing program

M. crucifer presented operational challenges for stakeholders. A method of rearing the agent in the laboratory was developed for initial releases and field assessments of establishment, dispersal and impact (De Clerck-Floate et al., 2005; De Clerck-Floate and Wikeem, 2009). However, a scaled-up version of this method for mass-production purposes was unfeasible, due to limited access to the space and the specialized personnel and equipment required to rear the weevil using greenhouse-grown houndstongue (Smith et al., 2009). The use of nursery sites was not feasible due to the difficulty of collecting the weevil, and because weevil populations do not persist and build up for very long before the houndstongue density becomes severely reduced, triggering dispersal (De Clerck-Floate et al., 2007). Similarly, *M. crucifer*'s habit of rapidly killing its host weed would not have made it well-suited for caged garden plot propagation. Instead, a relatively novel method was proposed for boosting the agent's numbers to meet a high, temporary demand; growing houndstongue as a field crop, then letting released *M. crucifer* propagate freely within the crop for two generations before being collected for use (De Clerck-Floate et al., 2007).

This 'farming project' (Fig. 6.2D) was launched as a proof-of-concept research study, with its focus on the development of easy, cost-effective methods for *M. crucifer* mass-rearing and collection that stakeholders could use (De Clerck-Floate et al., 2007). The project was funded by regional stakeholders. Their early involvement was welcomed, and there were instances of them providing helpful, practical solutions to challenges that arose during method development (e.g., the use of generator-powered, wet-dry vacuum cleaners to collect the weevils from propagation plots; Fig. 6.2E). During the study (2002−05), several agronomic practices for optimizing crop growth were experimentally varied, including seeding depth, rate, and time (spring or fall), crop fertilization, mulching, and methods of disease (powdery mildew) and annual weed control (De Clerck-Floate et al., 2007; Moyer et al., 2007). The experiment was replicated in time (twice within 2002−03 and 2003−04), and place (tilled fields in southeastern British Columbia and southern Alberta). Weevil population responses to all tested agronomic practices were monitored (Van Hezewijk et al., 2008).

There were technical challenges in method development. Foremost was figuring out a way to retain for collection the huge numbers of *M. crucifer* adults that emerged from the then-dead biennial houndstongue crop during the year of harvest (spring of third-year post-set-up). In the spring of the second year, rows of houndstongue distanced from the crop area were preplanted to serve as a green trap for emerging adults in the spring of the third year. However, while *M. crucifer* weevils were in their last round of replication during the second year, they also found and attacked the nearby trap plants, such that the plants were dead or dying by the time of weevil harvest. This prompted a quick replacement of dead trap plants with young houndstongue transplants during the harvest period (De Clerck-Floate et al., 2007).

6.4.2.2.2 Summary of the mass-rearing protocol

Agronomic practices that increased houndstongue root size were associated with boosted *M. crucifer* production during the farming experiment (De Clerck-Floate et al., 2007), likely because females prefer large plants for oviposition (Schwarzländer, 1997). This trend was especially evident in some site-years with the addition of nitrogen fertilizer to the houndstongue crop, at rates of either 50 or 100 kg/ha (De Clerck-Floate et al., 2007; Van Hezewijk et al., 2008). However, added nitrogen likely improved houndstongue quality in ways other than just size, as evident in a laboratory experiment in which plant size was kept constant, yet added nitrogen increased adult feeding by 24%, and oviposition by 25% (Van Hezewijk et al., 2008). Hence, the use of nitrogen fertilizer as a means to increase field production of *M. crucifer* and other root-feeding herbivores is recommended, particularly in cases where soil nitrogen levels are considered marginal, to begin with for annual crops (De Clerck-Floate et al., 2007).

A standard method of setting up the production field for *M. crucifer* mass-rearing was recommended for use by stakeholders, based on the results of the experiment (De Clerck-Floate et al., 2007; Moyer et al., 2007). This included the collection of sufficient houndstongue seed (nutlets) from existing field infestations of the weed ready for planting in the fall ahead of introducing *M. crucifer* to the houndstongue "crop" for mass-production. When planting the seed into a tilled or untilled field using a tractor-operated plot seeder, a row spacing of 67.5 cm, depth of 2 cm, and rate of 13 seeds m^{-2}

was recommended. Fertilizer application was recommended in the spring after planting, and herbicide treatment to keep annual weeds under control, as the herbicides had no significant effect on *M. crucifer* numbers. Inoculation of adult weevils into the houndstongue crop was recommended to take place in mid-summer of the year after planting (Year-1) to establish the first generation, which then was allowed to undergo a second generation on the same houndstongue crop in Year-2. In the fall of Year-1, houndstongue also was planted surrounding and within the existing crop to act as a trap for emergence in the spring of the third. year. Weevils emerged in masses from the soil in the midst of the now dead biennial crop, but were collected with a vacuum cleaner from the "green trap" plants (Fig. 6.2E). The vacuum cleaner contents were dumped into a closed, taped-up cardboard box (ca. 0.5 m^3), with a hole cut in one side as an escape for the weevils that crawl toward the light (Fig. 6.2F), as an effective, low-cost method to separate weevils from soil and debris. Fresh houndstongue leaves as bait was placed within a clear plastic bag, with one end taped around the box's hole before the other end was twist-tied shut. After 24 hours, the weevils were collected from the bags for counting, sorting and shipment.

6.4.2.2.3 Output of mass-rearing and estimated cost

The harvest from the two sites was estimated to be 162,000 M. *crucifer* weevils/ha (Smith et al., 2009). However, the adult harvest was thought to be only 10%−12% of the 1.6 million weevils/ha projected based on larval counts during the project, with the discrepancy thought to be partly due to mortality occurring before adult emergence, but also undoubtedly to the weevils dispersing in large numbers from the sites in search of their host. As mentioned, there were insufficient trap plants to attract and retain the weevils at the time of their mass emergence. *Mogulones crucifer* also is an excellent long-distance disperser and seeker of its host (De Clerck-Floate et al., 2005). An economic assessment of the costs associated with the farming method of mass-production, including the use of machinery, labor, fertilizer and other farm inputs, estimated a per weevil cost of CDN 0.10−0.14 (ca. US$ 0.08−0.11) in 2009 (Smith et al., 2009). In comparison, the estimated cost to rear the same insect at the same time in a research facility was CDN 2.65 (ca. US$2.12).

6.4.2.2.4 Impact of mass-rearing on establishment and efficacy

The thousands of *M. crucifer* collected from the field mass-rearing sites in 2004 and 2005 were distributed widely to stakeholders for release throughout the interior of British Columbia to control houndstongue. Many of the weevils produced at the southern Alberta site also found homes in the southeastern montane rangelands of the province, where houndstongue was the bane of local ranchers. As recommended from an earlier study on the optimum number of *M. crucifer* to release to achieve predictable, local control of houndstongue in southeastern British Columbia (De Clerck-Floate and Wikeem, 2009), weevils from the farming project were generally released in lots of 100/site. A return to many of these sites in 2009 to monitor establishment and impact left no doubt of the agent's success in the biological control of houndstongue, and as expected, the weevils continued to spread from established sites. Perhaps *M. crucifer* could have reached the same end goal after just a few initial strategic releases, but the novel field mass-rearing method certainly sped up the process.

6.4.3 South Africa

6.4.3.1 History of use

6.4.3.1.1 History of mass-rearing in biological control

The biocontrol of IAPs commenced in South Africa in 1913 when *Dactylopius ceylonicus* (Green) successfully controlled *Opuntia vulgaris* Miller (Hill et al., 2020). To date, 87 species of insects, mites and plant pathogens have been established on 66 IAPs species in the country (Zachariades, 2018). Until the mid-1990s, South African researchers conducted or oversaw most work on IAP biological control, including the mass-rearing, release and post-release monitoring of agents. This often worked well with a relatively high rate of establishment of agents, but for some agents (for instance, *Pareuchaetes* species on *C. odorata* (Zachariades et al., 2011)) establishment could only be achieved by large scale mass-rearing which was beyond the capacity of the research organization (Zachariades et al., 2017). With the establishment of the Working for Water (WfW) program in 1995, an 'implementation' program embedded within WfW was set up in the late 1990s (Gillespie et al., 2004), with the aims of mass-rearing; field collection for redistribution; releases and basic monitoring of establishment; and spread of BCAs. WfW considers the advancement of social and economic benefits for people as an essential element of environmental conservation (DEA, 2016). Short-term contract jobs are created for physical clearing activities but also biological and integrated control, with an emphasis on recruiting women (the target is 60%), youth (20%) and the disabled (5%) (DEA, 2016). Several mass-rearing centers were set up around the country. Currently, 47 BCAs for use against 33 IAPs are being mass-reared at eight facilities across the

country (examples in Table 6.1), providing approximately five million BCA individuals for release in South Africa annually (Zachariades et al., 2017) (examples in Table 6.1).

The objectives of setting up a mass-rearing facility were: To rear BCAs; to release reared BCAs into infested areas; to use the facility as a learning space; and, to use the facility to encourage public engagement and education around IAPs and biological control. Any mass-rearing program aims to produce the maximum number of good- quality insects with minimal labor and costs. The mass-rearing program in South Africa has been based on the hypothesis that continual releases of high numbers of insects will increase the likelihood of establishment and reduce the time between BCA release and control of the weed. Despite the advantages of releasing large numbers of insects, BCA quality should always be more important than BCA quantity.

6.4.3.1.2 Mass-rearing as employment opportunities

It has proven difficult to employ people with disabilities (PWDs) in WfW, as the majority of job opportunities are in daily-wage systems, which involve private contractors who employ laborers to clear existing IAPs manually. This work often excludes the majority of PWDs from employment. The development of two PWD-oriented mass-rearing facilities has provided opportunities for PWDs to be trained and employed in skilled positions, culturing and harvesting BCAs for release. Currently, the eight government-funded mass-rearing facilities in South Africa employ 32 personnel, of whom seven have physical disabilities (Zachariades et al., 2017).

6.4.3.1.3 Mass-rearing programs at schools

Mass-rearing in schools is possible for BCAs that are relatively easy to rear, collect, release and monitor with minimal effort, maintenance and infrastructure. School-based mass-rearing programs can be effective in the implementation of biological control as they can provide a direct link to infestations in, rural, less accessible areas (Briese and McLaren, 1997). Waterhyacinth (*Pontederia* (= *Eichhornia*) *crassipes* (Mart.)) Solms. (Pontederiaceae) provides a good example. In South Africa, despite extensive management initiatives, it remains one of the most problematic aquatic weeds (Hill and Coetzee, 2017). Biological control has been implemented since 1974, and although successful, BCAs need to be re-introduced regularly because the habitats in which the weed exists are prone to stochastic disturbances such as flooding, drought and herbicide application (Hill and Olckers, 2001). Several school-based BCA mass-rearing programs have been implemented. At each school, waterhyacinth is grown in lattice-wall ponds (Julien et al., 1999), and BCAs are introduced and re-collected for monitoring by school pupils. The program serves primarily as an educational tool for teachers to teach plant-insect interactions, ecology and ecosystem science (Weaver et al., 2017). These programs have proven successful; however, sustained inputs from university-based researchers are essential to sustain enthusiasm and infrastructure (Martin et al., 2018).

6.4.3.2 Case study: a leaf-feeding planthopper on waterhyacinth

The waterhyacinth planthopper *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) (Fig. 6.3A and B) is a phloem-feeding bug native to South America, as is its host. Adult females oviposit in the petiole and the lamina of waterhyacinth. Nymphs feed in phloem or less often in xylem tissues on the plant petioles close to the water surface (Hernández et al., 2011), by piercing the tissues of its host plant using a needle-like rostrum. Feeding causes direct damage and facilitates the entry of opportunistic plant pathogens (Sutton et al., 2016). Feeding results in a significant reduction in photosynthetic capacity (Miller et al., 2019). *Megamelus scutellaris* is a multivoltine species and immature stages overwinter in decayed mats of waterhyacinth (Sosa et al., 2005). Adults exhibit wing dimorphism, occurring as a long-winged form (macropterous) and a short-winged, nonflying form (brachypterous) (Fig. 6.3A and B). This is important for mass-rearing as the ratio of macroptery to brachyptery is determined by environmental cues such as crowding and host quality (Fitzgerald and Tipping, 2013). The planthopper was released first in the United States in 2010 (Goode et al., 2021; Tipping et al., 2014), and subsequently in South Africa in 2013 (Hill and Coetzee, 2017) but was not considered specific enough for release in Australia (Heard et al., 2014).

6.4.3.2.1 Key challenges in designing mass-rearing program

In South Africa, the main mass-rearing facility for *M. scutellaris* is the Center for Biological Control (CBC), Waainek Research Facility in Grahamstown/ Makhanda, Eastern Cape Province, where the insects are reared on plants growing in square plastic bins (1.2 × 1.2 × 1.2 m) housed in large (30 × 10 m) temperature controlled polyurethane tunnels (Fig. 6.3C). However, the insect is also reared on a much smaller scale at schools. Here the same bins are used, but the

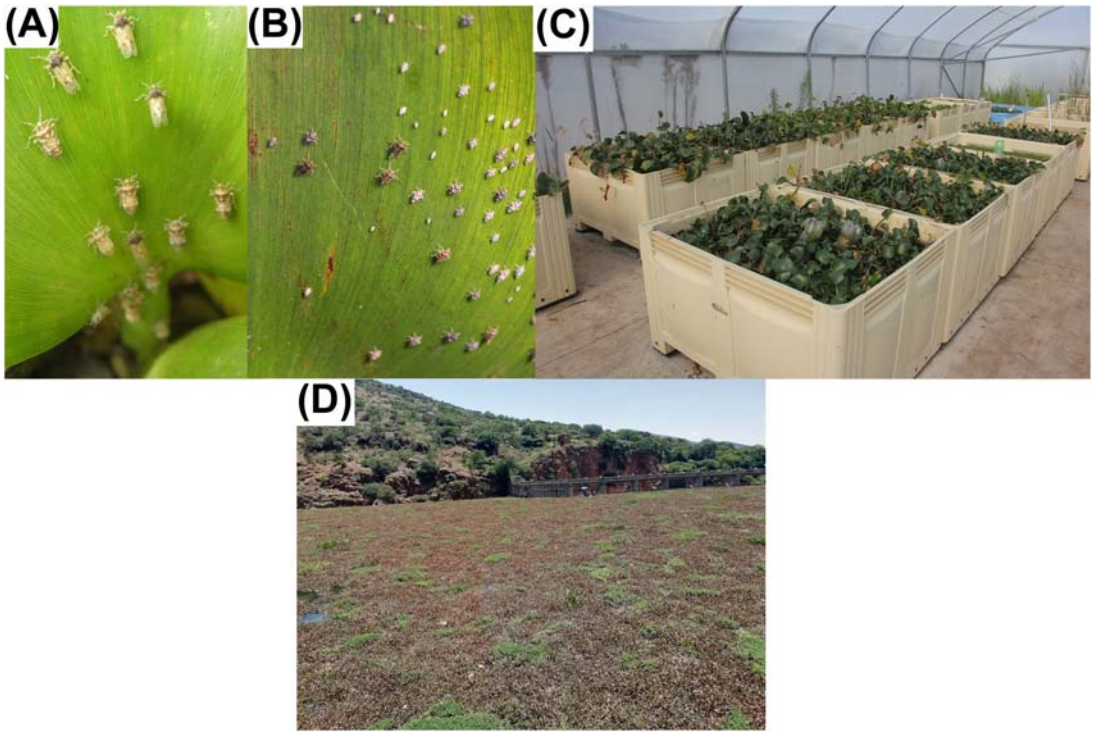

FIGURE 6.3 Mass-rearing and release of the waterhyacinth planthopper *Megamelus scutellaris* in greenhouse tanks in South Africa. (A) Macropterous and brachypterous adult forms. (B) Dense colony of nymphs and adults. (C) Rearing facility at the Centre for Biological Control at Rhodes University. (D) Waterhyacinth heavily damaged by the planthopper in early 2020 at Hartbeesport Dam, near Pretoria. Photo credits: (A), (D)-Julie Coetzee, Rhodes University; (B), (C)-Ben Miller, Rhodes University. (For a color version of this figure, the reader is referred to the online version of this book.)

tunnels are much smaller (6×3 m) and are not temperature-controlled. Factors considered in operating mass-rearing facilities for *M. scutellaris* include:

Physical: The rearing site needs to be sunny so that in winter it keeps the tunnel warm, but care should be taken that the plants and insects do not overheat in summer (temperatures above 40°C can be fatal over extended periods) (May and Coetzee, 2013). The optimal water temperature for healthy waterhyacinth growth is 25°C (Wilson et al., 2005). Access to an economical water supply needs to be considered, as well as access to nearby release points at weed-infested sites.

Maintenance: The facility at the CBC comprises a manager and seven technicians. The *M. scutellaris* component of the mass-rearing operations occupies 15% of the labor cost. The number of people required to maintain the facility should be considered in relation to its size to maximize efficiency. Paid (at the CBC) or volunteer (at schools) personnel needed to be selected, trained, and evaluated post-hire for their ability to record the density and conditions of the plants and BCA output frequently, such as weekly. Supervisory or project management oversight includes evaluation of changes in production efficiency or BCA quality over time.

Plant quality: The single most important determinant of *M. scutellaris* population increase under mass-rearing is plant quality (Fitzgerald and Tipping, 2013). Healthy cultures of *M. scutellaris* can reach high densities relatively quickly, so it is important to keep the plants healthy and to replenish plants that have been significantly damaged by BCA feeding. Field studies have shown that waterhyacinth plants that are healthy and have high tissue nitrogen concentration produce highly reproductive planthoppers and higher progeny yield than do poorer quality (low nutrient) plants (Sutton et al., 2016). Similarly, laboratory studies indicated that elevated nutrient content in high-quality plants can extend the duration of the reproductive period and increase total reproductive output of leaf-feeding weevils (*Neochetina* spp.) on waterhyacinth (Center and Dray, 2010) and increase their rate of larval development (Mukarugwiro et al., 2018). If the quality of plants used during rearing deteriorates, uncaged BCAs are likely to disperse to areas of the facility without food and the rearing process may collapse; or they may die out if caged. Nutrient levels influence the growth rate and biomass of water hyacinth (Wilson et al., 2005), which in turn influence the rate of population increase of *M. scutellaris*. Weekly water nutrient and pH testing are required to ensure that the nutrient level

is sufficient to promote waterhyacinth growth. A water soluble fertilizer that is high in nitrogen (N:P:K-6:1:3) is added monthly at a rate of 56 g/1000 L to stimulate growth of foliage. During winter, when the plants grow more slowly, this rate is increased to 76 g/1000/L per month to counter pest pathogen and mite buildup. Micronutrients are added in a chelated form, making this an ideal fertilizer for foliage production. Standard greenhouse pests such as aphids and mites can only be controlled before introducing plants to the *M. scutellaris* colony tanks, and only using physical methods (such as water spray or high temperature) or organic chemical methods (like dilute soap). Monitoring and adjustment of pH and nutrient levels to avoid over-fertilization, agitation of water and dyes are used to control algae.

Temperature: The genotype of *M. scutellaris* that has been released in South Africa has a small thermal window for development. The lower developmental threshold is 11.45°C and no development takes place over 30°C (May and Coetzee, 2013). Thus, at least some temperature control in the mass-rearing facility (tunnels) is essential to allow a high or maximum rate of increase and avoid crashes. The facility at the CBC is located in a cool temperate region of the country where minimum temperatures during winter drop below the lower thermal threshold of but rise inside the tunnels above 30°C in the summer, and thus the rearing facility is heated during winter and cooled during summer to ensure a temperature range of 16°C and 26°C.

Parasitism: Recently, the indigenous wasp species *Echthrodelphax migratorius* Benoit, (Hymenoptera: Dryinidae) was discovered in South Africa parasitizing *M. scutellaris* nymphs and adults, both in the field and the mass-rearing facilities (Kraus et al., 2019). It is not known what impact this parasitoid will have on the mass-rearing of this agent. A similar parasitoid has been recorded in Argentina (Sosa et al., 2005) and in Florida, United States (Minteer et al., 2016). Besides rearing under gauze and avoiding contamination from field populations, there is no practical way to eradicate the parasitoid from rearing facilities.

Collecting techniques: Rearing tunnels are designed to accommodate the access needs of PWDs. It was difficult for staff to reach the middle of the 3 m diameter pools initially used, and so the facility was refurbished with the smaller bins described earlier (Fig. 6.3C). A further development was the introduction of battery-operated aspirators to collect the BCA, rather than the traditional human-powered ones. These improvements massively increased the number of insects collected and made a positive contribution to occupational health and safety.

Contamination with other BCAs: The mass-rearing facility at the CBCis not dedicated solely to mass-rearing of *M. scutellaris*. Other BCAs growing on waterhyacinth and other aquatic weeds, including on *Salvinia molesta* D. Mitch, *Pistia stratiotes* L. and *Myriophyllum aquaticum* (Velloso) Verdcourt are present in nearby tanks, raising the potential for contamination. In particular, two mites targeting waterhyacinth, *Eccritotarsus catarinensis* (Carvalho) and *E. eichhorniae*, often get collected in the same sample. This is not overly problematic as they are compatible with *M. scutellaris* (Goode et al., 2020).

Summary of the mass-rearing protocol. The CBC rearing facility comprises three of the temperature-controlled polyurethane tunnels described before, of which one is dedicated to the mass-rearing of waterhyacinth control agents. The tunnel temperature is maintained between 16°C in winter and 26°C in summer and only natural light is provided. Plants are grown in square plastic bins (1.7 m^3) and the bins are left uncovered (Fig. 6.3C). Water soluble high-nitrogen fertilizer (N:P:K—6:1:3) is added at the seasonal rates noted above for summer (56 g/1000/L) and winter growth conditions (1.4 × that rate). The water in the bins is drained and replaced annually. Fifty adult *M. scutellaris* are added to each of the bins and maintained for 30 days. After this period harvesting occurs two days per week, every week during summer and every two weeks during winter. Once the insects are collected, they are placed into insulated boxes (30 × 30 × 20 cm) lined with paper toweling to reduce humidity. Approximately 5000 individuals (both adults and nymphs) are packed in each container and fresh waterhyacinth leaves are provided to sustain them during transport. The consignments are then posted to landowners and implementing agents by 24-hour postal service.

6.4.3.2.2 Output of mass-rearing and estimated cost

Over a 7-year period about 944,000 adults and nymphs of *M. scutellaris* have been mass-reared and released against waterhyacinth at 30 sites (149 release events) around South Africa (Table 6.2). The total cost for the 2019 year including labor, materials and postage of insects was ZAR 188,550 (ca. US$13,184).

6.4.3.2.3 Impact of mass-rearing on establishment and efficacy

Megamelus scutellaris has established at 17 sites throughout South Africa, and it has dispersed to an additional four sites. At the remainder of the release sites, the insect has failed to establish (six sites), or the sites have not been revisited (seven sites). The insect has established in cool temperate regions, coastal Mediterranean type climates and warm subtropical regions and thus there appears to be no thermal limitation on this insect in the field in South Africa

TABLE 6.2 The number of *Megamellus scutellaris* planthoppers reared and released against waterhyacinth in South Africa between 2014 and 2020.

Year	Number released	Number of releases[a]
2014	41,000	19
2015	251,900	43
2016	125,000	20
2017	58,000	7
2018	143,000	24
2019	228,000	24
2020	98,000	12
Total	**944,200**	**149**

[a]*Planthoppers were sent to many sites on more than one occasion.*

(Miller et al., 2019, 2021). The insect failed to establish at sites where there was interference from other management practices such as herbicide application (four sites) and at two sites at which low (>1000) numbers were released.

A recent example of the benefit of the mass-rearing program for *M. scutellaris* comes from a highly eutrophic impoundment in the high elevation (>1200 m) cool temperate region. The Hartbeespoort Reservoir is in the North-West Province of South Africa, about 30 km west of Pretoria, the capital of South Africa. The reservoir catchment is approximately 4100 km^2 and extends southwards from the dam, incorporating the Crocodile River and Magalies River, as well as minor, nonperennial streams (Walmsley et al., 1978). Daily temperatures average 5°C−24°C in winter (May to July) and 16°C−30°C in summer (November to January). Frost occurs on winter mornings but is uncommon. The rainy season commences in October and extends until March or April, and the mean annual rainfall is 670 mm. Winter (dry season) flows are larger than natural flows due to urban runoff and sewage inputs. Hartbeespoort Dam supplies irrigation water but deteriorating water quality, caused most notably by microcystin toxin, is causing much of the irrigated agriculture to fall into disuse (Du Preez et al., 2018). It also supplies water for domestic and industrial use, and the reservoir is used for recreation.

The reservoir has been hypertrophic since the 1970s as a result of upstream urban and industrial pollution, and as a result, it is a hotspot of waterhyacinth invasion. The presence of this weed reduced recreational usage as well as water light and dissolved oxygen, limiting the growth of submerged plants and other organisms, with trophic effects on organisms that feed on them (Ashton et al., 1979; Cilliers, 1991). The plant was successfully controlled starting in the 1980s using herbicides. In 2016, however, herbicidal control was halted. By the end of 2017, more than 50% of the surface was covered. Mechanical removal and an inundative biological control program were implemented in 2018. Site inspections revealed good population densities of the *Neochetina* weevils, as well as the mite, *Orthogalumna terebrantis*, both released in the 1990s (Cilliers, 1991), which had persisted despite the spraying program. The CBC embarked on extensive mass-rearing and releases of *M. scutellaris*. In 2019, between 8000 and 10,000 hoppers were released onto the system every two weeks through the winter period. Establishment was confirmed in late winter (August 2019), and by the following summer (December 2019), cover was below 20%. At the end of January 2020, inspections by CBC researchers found that less than 7% of the surface was covered, and the remaining waterhyacinth plants were brown and heavily damaged (Fig. 6.3D). Release of the planthopper, along with increases in populations of the other two agents to levels not encountered in the field before, resulted in the collapse of the water hyacinth mats and complete control of waterhyacinth, equivalent to the prior chemical control program.

6.4.4 Australia

6.4.4.1 History of use

The spectacular success of the classical biological control of prickly pear (*Opuntia* spp.) in Australia at the turn of the 20th century launched weed classical biological control in Australia, and globally, as a viable and sustainable method of landscape-scale weed management (Walton, 2005). Key to success was the amazing mass-rearing and release efforts

(Raghu and Walton, 2007). Since those initial successes, there has been a steady legacy of benefits of classical biological weeds accruing in Australia, with over 202 agents (including pathogens) released and 56 weeds targeted (Schwarzländer et al., 2018; Winston et al., 2021). Economic analyses conservatively place the average value of these benefits to be 23 times the investment in the development of BCAs (Page and Lacey, 2006; van Wilgen et al., 2020).

In Australia, contemporary investment in the establishment of long-term mass-rearing and release/ "implementation" programs has often had limited funding support. Research agencies involved in developing classical BCAs have largely been responsible for the initial phases of rearing and release. In cases where there is a subsequent investment (typically from the state- and local-government level), research agencies facilitate technology transfer to regional and local agencies. Technology transfer includes details on the biology of targeted weeds and agents, the actual BCA(s), optimal methods for release to enable BCA establishment, and an outline of how best to evaluate BCA performance (for instance population dynamics, natural spread to sites where releases have not occurred) and their impact on the target weed. Recipient agencies include state/territory-based extension agencies, industry groups, nongovernmental organizations, and community groups (Briese and McLaren, 1997).

The establishment of mass-rearing hubs for weed BCAs in Australia has been variable based on the scale of the target weed's distribution and the contemporaneous availability of a BCA approved for release. In some instances, mass-rearing efforts are directly supported by state government research agencies. The Queensland, New South Wales, and Victorian governments (all undertaking research to develop BCAs) have maintained mass-rearing and release operations (NSWDPI, 2019). Regional land care and local government agencies have also been critical in mass-rearing efforts. For example, Gympie District Landcare (https://gympielandcare.org.au/services/bio-control-weeds/) has been a vital regional partner in the mass-rearing and release of agents targeting several terrestrial environmental weeds. Similarly, Brisbane City Council (https://www.brisbane.qld.gov.au/clean-and-green/natural-environment-and-water/biodiversity-in-brisbane/wildlife-in-brisbane/pest-animals-and-invasive-species/biocontrol-for-aquatic-weeds) has maintained a mass-rearing facility for aquatic weed BCAs for the past two decades. This facility has been crucial to the releases of BCAs on *Salvinia molesta* Mitchell, *Pistia stratiotes* and *P. crassipes* across multiple catchments in south-eastern Queensland. Some small-scale commercial mass-rearing operations periodically emerge in Australia (http://www.weedbiocontrol.com.au/).

Despite over a century of weed classical biological control in Australia, no other biological control program has achieved a mass-rearing release and monitoring effort comparable to that of prickly pear (Raghu and Walton, 2007) (Table 6.1). In the recent past, efforts focused on BCAs for pasture weeds in temperate Australia between 1990 and the early 2000s represents one of the best examples (CRCWMS, 1998; Agtrans Research, 2002). In the case of environmental weeds, the program targets the biological control of bridal creepers across Western Australia, South Australia and New South Wales (Batchelor and Woodburn, 2002; Morin et al., 2002, 2006) is a noteworthy example. More recently, the Rural Research and Development for Profit scheme (funding projects from 2015−22) of the Commonwealth of Australia incentivized closer collaborations between research agencies and end-users of biological control. This has enabled opportunities for better translation of biological control research into mass-rearing and release efforts (Allan, 2018), including the establishment of geospatial digital platforms to enable greater citizen science engagement (https://biocollect.ala.org.au/biocontrolhub). A recent case study that exemplifies such collaboration is on the mass-rearing and release efforts related to the neotropical weed parkinsonia (*Parkinsonia aculeata* L., Leguminosae); in addition to collaborations, this case study also highlights how contemporary climate modeling approaches have optimized release efforts to give the agents the best chance of establishment and impact across northern Australia.

6.4.4.2 Case study: two leaf-feeding moths on Parkinsonia

Native to the Americas, Parkinsonia (*P. aculeata*) was introduced into Australia as an ornamental tree/shrub and for its potential value for hedging and as fodder (Hawkins et al., 2007). Inhabiting semi-arid and tropical rangelands across northern Australia and the Kimberley and Pilbara regions of Western Australia, its current distribution extends over some 8000 km^2 (Deveze, 2004; van Klinken et al., 2009; van Klinken and Heard, 2012), with potential for further range expansion into suitable habitat. It has the ability to form dense thickets in floodplains and grasslands along watercourses and bore drains, thereby negatively impacting the pastoral industry (for example limiting pasture growth, restricting stock access to water and impeding mustering) and the environment (like providing refuges for feral animals like pigs, increasing evapotranspiration, contributing to soil erosion, suppressing the herb layer and reducing wildlife habitat). Parkinsonia is a declared weed in all states and territories of Australia, is considered a Weed of National Significance and has been a target for biological control in Australia since 1983 (Deveze, 2004; van Klinken and Heard, 2012).

Based on an ecological understanding of its population dynamics, and consultations with key management stakeholders, the goals of an integrated weed management program for landscape-scale control of Parkinsonia were devised (Deveze, 2004; Raghu et al., 2006; van Klinken, 2006; Pichancourt and van Klinken, 2012; Pichancourt et al., 2019). These included (1) reducing patch density (<30% cover) and size (<1 ha); (2) reducing rates of spread and in-fill by reducing seed production/density (<100 viable seeds/m^2); (3) reducing growth and recruitment (by 50%) and delay time to reproduction (by 1 year; currently 2–3 years); and (4) targeting management in regions at highest risk from Parkinsonia impacts. Biological control's role in the integrated management of Parkinsonia will best be judged by its ability to slow plant vigor and reduce seed production (directly, and indirectly through impacting nonreproductive life stages), and the extent to which it can consequently limit the growth and spread of Parkinsonia populations (Raghu et al., 2006; van Klinken, 2006; Pichancourt et al., 2019).

Research by Queensland Government researchers on biological control of Parkinsonia resulted in the introduction of three insect species between 1989 and 1995: a sap-sucking bug (*Rhinacloa callicrates* Herring), and two seed-feeding beetles (*Mimosetes ulkei* (Horn), and *Penthobruchus germaini* Pic). These BCAs have been inadequate on their own to control Parkinsonia populations. New surveys were conducted by CSIRO in 2002 across central and South America, including in Argentina, Brazil, Costa Rica, Guatemala, Mexico, Nicaragua, Paraguay, Peru, the USA, and Venezuela (van Klinken, 2006; van Klinken and Heard, 2012). Based on detailed tests to demonstrate their safety, CSIRO received approval from the Commonwealth of Australia, in 2012 and 2014,respectively, to release two closely related leaf-feeding moths, *Eueupithecia cisplatensis* Prout and *Eueupithecia vollonoides* Hausmann (Lepidoptera: Geometridae) (Hausmann et al., 2016). Through funding from the Rural Research and Development for Profit scheme and Meat and Livestock Australia, and a network of some 20 collaborating partner agencies, spatially extensive releases of these agents were achieved across northern Australia.

6.4.4.2.1 Key challenges in designing mass-rearing program

Eueupithecia cisplatensis (UU1) and *E. vollonoides* (UU2) are leaf feeders and have a similar life history. The female moth lays eggs (Fig. 6.4A) on the leaves of Parkinsonia. Development of the moths at a temperature of 25°C–28°C has the following timelines. Eggs hatch after 5–7 days and newly hatched larvae, less than 2 mm long, begin feeding on the leaves (Fig. 6.4D). The larvae continue feeding for around 15 days and grow to approximately 2 cm in length after four stadia (Fig. 6.4B) before pupating. Adult moths (Fig. 6.4C) emerge from cocoons after 5–7 days. Larvae and adults of UU1 and UU2 are outwardly similar and can be morphologically distinguished only by dissection and examination of their genitalia. Despite similarities in biology, the two species have slightly different environmental requirements in their native range (Hausmann et al., 2016), with UU1 occurring in the coastal, slightly cooler and more humid southeast of Argentina, and UU2 occurring in the inland, hotter and drier northwest of northern Argentina.

A significant challenge in this project was the logistics of shipping larvae, given that Parkinsonia occupies some of the most remote regions of northern Australia away from population centers, with challenging access. While it is easy to rear and pack large numbers of larvae (Fig. 6.5A) or pupae (Fig. 6.5B), larvae are vulnerable to heat stress in packaging and poor handling in transit (like packaging left in the sun by mail/courier agencies). This can result in them reaching remote release sites in a poor/sub-optimal condition that lowers their odds of survival. Larvae have the capacity to seek suitable microclimates amid the plant canopy when released in the field. However, the release of large numbers of larvae can attract native predators (such as ants, wasps, reptiles and birds) to the release site, resulting in potentially

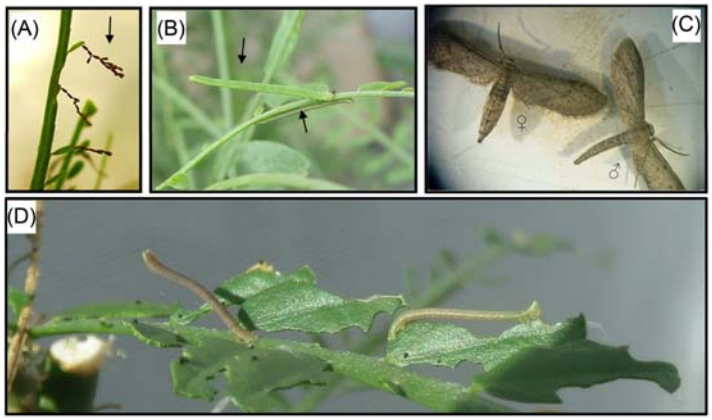

FIGURE 6.4 Morphology of life stages of *Eueupithecia cisplatensis* (UU1). (A) Eggs (indicated by arrow). (B) Larvae (indicated by arrows). (C) Adults. (D). Damage by larvae on leaves. Both UU1 and UU2 (*Eueupithecia vollonoides*) have a similar appearance throughout their life cycle. With experience, the larger size of UU2 adults relative to UU1 is apparent. (For a color version of this figure, the reader is referred to the online version of this book.) *Photo credits: CSIRO.*

FIGURE 6.5 Mass-rearing of *E. cisplatensis* (UU1) and *E. vollonoides* (UU2) at nursery field sites in Australia. (A) Shipment box of larvae. (B) Shipment box of pupae. (C) and (D) Releases of larvae into a Parkinsonia "nest." (E) Setting up a pyramid shelter for release of pupae. (F) Coating the shelter's handle with Tanglefoot™ to prevent ant predation of pupae. (G) Container of pupae placed in pyramidal shelter, showing adult UU1 emerging). (H) Beat sheet method for detection of dislodged UU1/UU2 larvae. *Photo credits: (A, C–H)—CSIRO; (B)–Kelli Pukallus, QDAF.*

significant mortality. Releasing in protected Parkinsonia "nests" (Fig. 6.5C and D) was an important way to minimize this risk, but highly mobile predators like ants may still have had the potential to cause significant mortality of agents and impact agent densities in the field. Release of adults was another option, but adults of these moths are short-lived and are equally prone to the vulnerabilities of shipment as larvae. This problem could be overcome through the release of pupae. Adults emerging from pupae were expected to mate under field conditions and find oviposition sites that may have a suitable microclimate for larval development.

6.4.4.2.2 Summary of the mass-rearing protocol

UU1 and UU2 were mass-reared at the CSIRO facilities at the Ecosciences Precinct in Brisbane, and Queensland's Department of Agriculture and Fisheries' Tropical Weeds Research Centre, Charters Towers. In addition, UU1 was mass-reared by the Northern Territory Department of Land Resources Management's Weeds Branch in Darwin. Rearing was done under optimal environmental conditions for the plant and the two insect species. Eggs laid by female moths were maintained in the laboratory until neonates hatched; these were then transferred onto the leaves of Parkinsonia plants growing in cages in an air-conditioned greenhouse (ca. 25°C–28°C; 50%–60% RH). After completing development newly emerged adults were collected daily from colony cages and paired with adults emerging from different cages (to ensure adequate genetic diversity and limit the likelihood of inbreeding). These mating pairs were confined in plastic containers (17 × 11 × 5 cm) to ensure mating and oviposition. Containers were lined with moistened power towels to maintain high humidity and prevent the desiccation of eggs. Eggs were either returned to colony cages or were lab-reared in anticipation of field release.

Lab rearing involved maintaining the eggs in plastic containers in a lab environment (25°C–28°C; 50%–60% RH), after removing adults. Upon hatch, neonates were presented with healthy sprigs of Parkinsonia leaves as food and fresh sprigs of leaves were supplemented regularly to ensure that a density of up to 200 larvae could be maintained in each container. Larvae were typically shipped or transported to the release location on sprigs of Parkinsonia (Fig. 6.5A), while pupae were packed with dry paper towels (Fig. 6.5B). Shipments of larvae/pupae to release sites were made in polystyrene cooler boxes with ice bricks to prevent extreme temperatures during transit and enable safe delivery to remote locations, with transit times of up to a week.

In each state/territory, we identified several locations to serve as "nursery sites" for each of the two species. Selection of optimal nursery sites was guided by the following features: (1) Parkinsonia plants were in healthy condition, most typically when growing as part of riparian vegetation, or on the bank of a dam/reservoir. (2) The sites were easily accessible to enable regular releases of the insects and were not earmarked for other management (such as mechanical or chemical control) in the near future. (3) Plants did not show signs of sooty mold or scale insects. The latter was usually a good indicator of the presence of ants that are a major predator of these BCAs.

The use of nursery sites was important to ensure reliable establishment of BCAs. Over time, established populations would spread and colonize other sites. Larvae were released in "nests" of shoots tied together (Fig. 6.5C and D). This practice maximized the chances of survival for the larvae by providing abundant food, and shelter from predators (such as ants, wasps, reptiles, and birds). Containers with pupae were housed in pyramidal shelters during their release (Delta Traps, ISCA Technologies Inc., Riverside, CA, United States) (Fig. 6.5E–G), and these shelters were suspended from a Parkinsonia branch using twine or a cable. A nontoxic glue (Tanglefoot™, The Scotts Company LLC, Marysville, OH, USA) was applied to the twine/cable to prevent ant predation (Fig. 6.5F). Field collections of larvae for redistribution were done using beat-sheet methods (Fig. 6.5H). Over the course of 5 years, over 20 and 10 nursery sites were established for UU1 and UU2, respectively, across Queensland, Northern Territory and Western Australia.

6.4.4.2.3 Output of mass-rearing and estimated cost

Field releases in this project were principally focused on larvae, although, on occasion, pupae were released. A nationally-coordinated field release program was developed in discussion with key collaborators in Queensland, Northern Territory and Western Australia (Fig. 6.6). All collaborators received a detailed dossier outlining the biology of the BCAs, methods to rear the moths, characteristics of optimal sites for release, and a methodology to monitor the establishment and spread. Details of releases (including GPS coordinates, photos, dates and number of insects released) were recorded on a standardized data sheet by collaborators doing the field releases and returned to the project team.

More than a million individuals of both moth species at over two hundred parkinsonia infestation sites were released (Table 6.3). Releases outside of nursery sites were made on pastoral properties; these were either made directly by the project team or by pastoralists and regional biosecurity officers receiving the BCAs from the research agencies managing colonies, or from direct field collection of larvae from the nursery sites. The average numbers of insects released at nursery sites of UU1 and UU2 were 41,672 (median = 21,945) and 25,852 (median = 25,635), respectively. For the nonnursery sites, the average release numbers were 4265 and 4370 per site for UU1 and UU2, respectively. The overall cost of the Parkinsonia biological control program since 2002 has been estimated to be ca AU$8 million (ca. US$6 million), with the mass-rearing and release of UU1 and UU2 estimated to cost ca AU$1 million (ca. US$750,000).

Bioclimatic modeling to guide and evaluate the release effort: The selection of release sites for this project was guided by an understanding of the bioclimatic suitability of UU1 and UU2. The bioclimatic envelope. that is, the predicted species distribution model (Čengić et al., 2020), of each of the two BCAs was modeled using MaxEnt based on their known distribution in the native range, and these were projected onto the Parkinsonia infestations in Australia. Releases were then structured to include sites with characteristics fitting all the different suitability classes identified by the models (Fig. 6.7). Undertaking releases in this manner, in addition to enabling establishment, ensured that the value

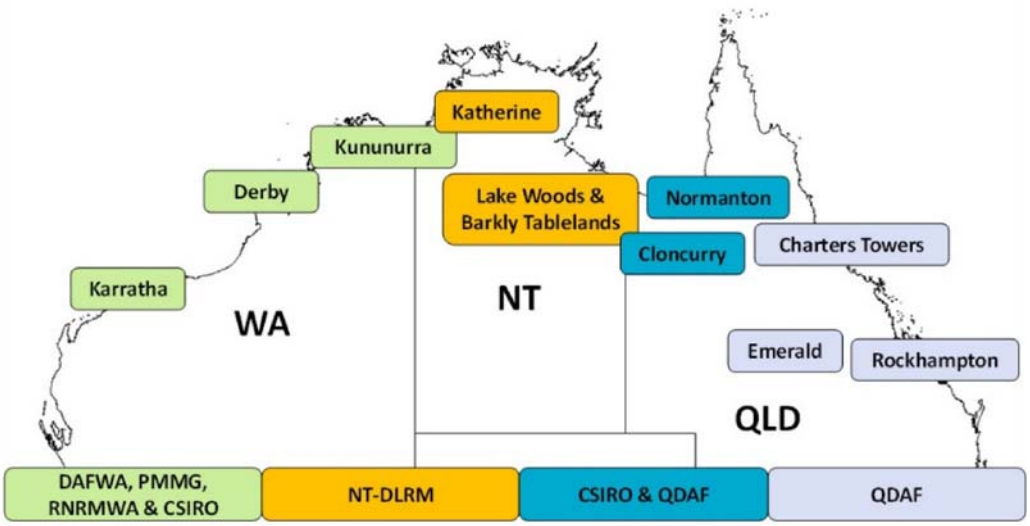

FIGURE 6.6 Coordinated release of *E. cisplatensis* and *E. vollonoides* across parkinsonia infestations in northern Australia was enabled by key collaborations in Queensland (QT) [Department of Agriculture and Fisheries (QDAF)]; Western Australia (WA) [Department of Agriculture and Food WA (DAFWA)]; Pilbara Mesquite Management Group (PMMG); Rangelands Natural Resource Management of WA (RNRMWA); and Northern Territory [Department of Land Resources Management (NT-DLRM)]. Agency names were current at the time the work was performed.

TABLE 6.3 Summary of releases made, between 2014 and 2019, of the two *Eueupithecia* spp. across Northern Australia.

Species	State	No. of sites	No. of releases	Total pupae released	Total larvae released
Eueupithecia cisplatensis	QLD	150	365	324,190	599,098
(UU1)	WA	12	25	15,624	71,520
	NT	26	50	33,577	48,722
	Total	**188**	**440**	**373,391**	**719,340**
E. vollonoides	QLD	26	53	28,829	145,200
(UU2)	WA	8	28	7197	97,200
	NT	8	12	10,570	1900
	Total	**42**	**93**	**46,596**	**244,300**

QLD, Queensland; *NT*, Northern Territory; *WA*, Western Australia.

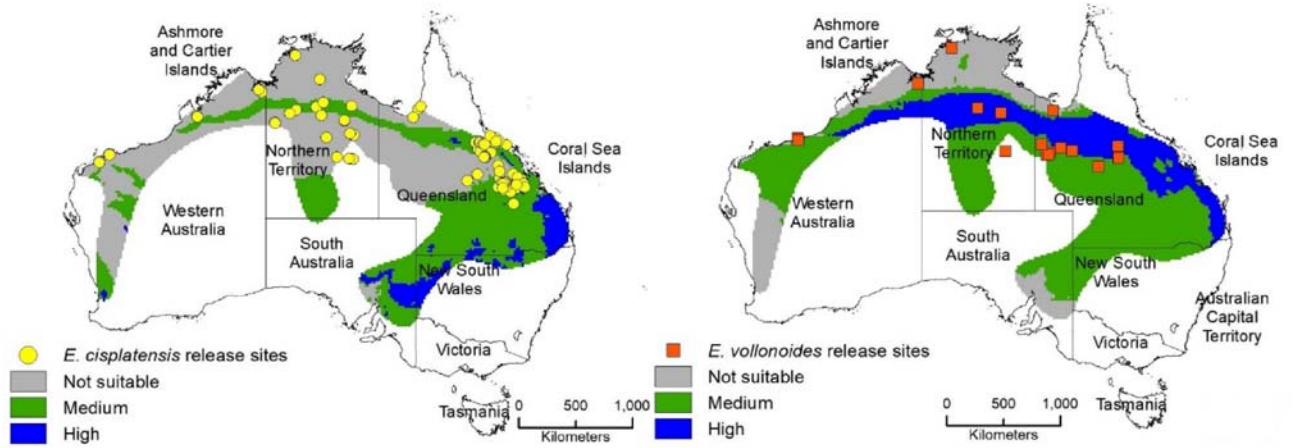

FIGURE 6.7 Distribution of release sites of *E. cisplatensis* (UU1) and *E. vollonoides* (UU2), as of 2019, in relation to bioclimatic suitability. The current Australian distribution of Parkinsonia (region shaded in gray, as captured by a minimum convex polygon of distribution records) and bioclimatic suitability projections for *E. cisplatensis* (UU1, left) and *E. vollonoides* (UU2, right) as revealed by species distribution modeling using MaxEnt. Suitability was defined as "Medium" when less than 50% of the iterative models predicted that a particular grid cell on the map has a suitable climate for the species, and as "High" if more than 50% of the models predicted a suitable climate. Projections are at 1-degree grid resolution.

of such bioclimatic modeling efforts to predict establishment was tested and became applicable to BCAs targeting other weeds in the future.

6.4.4.2.4 Impact of mass-rearing on establishment and efficacy

The use of nursery sites to optimize the establishment of UU1 and UU2 was not novel; such approaches are regularly used in releases of BCAs. However, the use of bioclimatic models to guide the selection of nursery and release sites was particularly useful for this widely-distributed weed. Using this approach, we have been able to release substantial numbers of the two moths across a vast area. Monitoring surveys have revealed that BCA populations had established at over 95% of the nursery sites, validating our release approach. Populations of the agents have begun to spread out across the landscape and at least one of the BCAs has been detected at 33 of the 56 release sites (~60%) to date.

Parkinsonia health and reproductive output are impacted by the ability of UU1 and UU2 to defoliate plants (Fig. 6.4D). This defoliation results in reduced growth rates, thereby delaying maturation and reducing the plant's ability to allocate resources to reproductive output. Studies on the impacts of feeding by larvae of these moths on Parkinsonia show that a larval density of 10 or more per juvenile plant can reduce canopy cover by approximately 60% and slow the growth rate of the plant (Raghu et al., 2017). Surveys for population establishment suggest that such larval

densities are being achieved in the field (albeit variably) by both UU1 and UU2 (Raghu et al., 2017; Allan, 2018), with significant feeding damage at some sites. Past demographic studies indicate that the growth rate of juvenile, pre-reproductive plants must be impacted for effective Parkinsonia control (Pichancourt and van Klinken, 2012; Pichancourt et al., 2019; Raghu et al., 2006). UU1 and UU2 can have 6−10 generations per year under the climate typically experienced by Parkinsonia infestations in northern Australia. Hence, repeated defoliation and significant impacts to juvenile plants and, over time, to local mature Parkinsonia populations can be anticipated if adequate densities of BCAs are maintained (Raghu et al., 2006; Pichancourt and van Klinken, 2012; Pichancourt et al., 2019).

If the impacts of defoliation outlined above are replicated across 50% of the total Parkinsonia infestation area over the next decade, it could help to reduce current recurring annual weed management costs by 10% and improve pasture productivity by AU$1−2/ha/y (ca. US$0.7−1.5/ha/y). This would give the biological control program an estimated benefit cost ratio of ca. 3.44 (Raghu et al., 2017). We intend to test/validate the assumptions of these analyses against field performance and the impacts of the agents in the years ahead.

6.4.5 New Zealand

6.4.5.1 History of use

The first weed BCA to be released in New Zealand was the cinnabar moth *Tyria jacobaeae* (L), in 1929, and it was mass-reared in very large numbers: between 1929 and 1932 over 3.4 million eggs were distributed throughout the country (Miller, 1970).

The second agent to be released was the gorse seed weevil *Exapion ulicis* (Forster), which was difficult to rear in containment. Only a relatively small number (560) of laboratory-reared beetles was released in 1931, augmented by the release of about 38,000 individuals imported from England (Paynter et al., 2016), followed by redistribution from established sites (Hill and Gourlay, 1989). The next three agents to be released (*Botanophila seneciella* (Meade) in 1936; *Chrysolina hyperici* Forster in 1943 and *Procecidochares utilis* Stone in 1958) were all released directly into the field following importation in relatively large numbers (for example, ca. 151,500 *B. seneciella*), with no attempt to rear them.

Since 1961, most weed BCAs were reared for release apart from a few species that could not be reared, for example, because they did not mate in captivity (Paynter et al., 2016). In 1984, a regulatory requirement for BCAs to be reliably screened for the presence of pathogenic organisms prior to release from containment was enacted, which constrained the number of agents that could be processed for direct field releases (Paynter et al., 2016). Overall, 52 weed biocontrol agents were released in New Zealand between 1929 and 2015, of which 43 (83%) became established. Of these, 43 were reared and released in numbers that ranged from just 173 to 3.4 million (median 19,000) resulting in 38 established BCAs (88%). Nine species were released directly into the field without being reared (range: 10−235,000; median 580), resulting in five establishments (55%). Paynter et al. (2016) showed that the probability of establishment was positively correlated with the number of individuals released although the threshold number for a high probability of establishment was relatively low. For example, eight out of nine BCA releases that became established that involved only 501−5000 individuals. The main benefit of mass-rearing was therefore to ensure that a BCA was rapidly established throughout a weed species range. Nevertheless, it has been common practice in New Zealand to redistribute agents by collecting stock from early release sites to minimize costly, labor-intensive rearing efforts. For example, the nodding thistle crown weevils *Trichosirocalus horridus* (Panzer) established following a release of only 200 individuals (Jessep, 1989) and were subsequently redistributed using field-collected individuals.

Weed biocontrol programs conducted since 2002 have been funded by the National Biocontrol Collective (regional councils nationwide and the Department of Conservation) together with the Ministry for Primary Industries via grants to interest groups from its Sustainable Farming Fund (Hayes et al., 2013). The National Biocontrol Collective funds the development of biocontrol agents (i.e., from initial feasibility studies, field surveys and host-specificity testing though to making petitions to release agents). Rearing of approved agents is funded by selling agents to stakeholders on a cost-recovery basis, which has ensured that BCAs are widely released but has imposed some constraints on the numbers reared as demand is relatively limited.

6.4.5.2 Case study: a leaf-feeding butterfly on Japanese honeysuckle

The Honshu white admiral butterfly *Limenitis glorifica* Fruhstorfer (Lepidoptera: Nymphalidae) is endemic to the island of Honshu, Japan. It is widely distributed from the western lowlands of Yamaguchi Prefecture to Shimokita Peninsula in the north (Fukuda et al., 1983). In central Honshu, there are typically three generations from mid-May to late

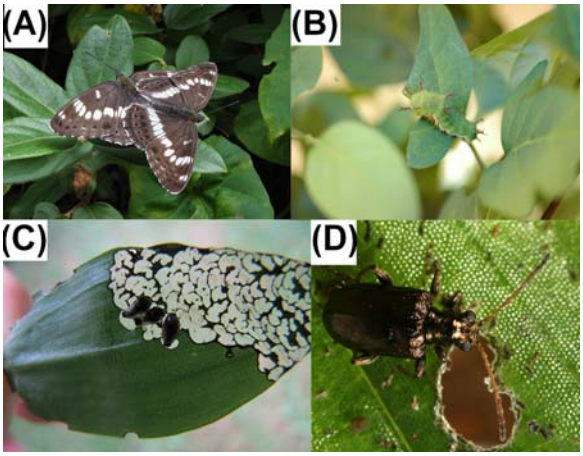

FIGURE 6.8 Two mass-reared biological control agents were released and recently established in New Zealand. (A) Larva of the Honshu white admiral butterfly *Limenitis glorifica*, released for biological control of Japanese honeysuckle. (B) Mating adults. (C) Larvae of the tradescantia leaf beetle *Neolema ogloblini* and (D) adult. *Photo credits: Q. Paynter, Landcare Research.*

September, but in late October a fourth-generation sometimes occurs. In cold mountainous zones, there may be only one generation each year. The adults (Fig. 6.8A) live for up to a month and the females produce 150−200 eggs, which are oviposited singly on Japanese honeysuckle (*Lonicera japonica* (Thunb.)) leaves. The larvae feed externally on the foliage. The early larval instars are brown and rest on characteristic "pier-like" extensions to the midrib of leaves that they make from silk and frass. Later instars (Fig. 6.8B) are green with several spiky, brown, horn-like protrusions. Larvae suspend themselves upside down, prior to pupation on the plant or surrounding vegetation. Development from egg to adult can occur in as little as 6 weeks. Overwintering occurs as a third instar within a hibernaculum made from the base of a leaf folded over and held together with silk.

6.4.5.2.1 Key challenges in designing mass-rearing program

Host-specificity testing of *L. glorifica* was conducted in Japan using field-collected material because butterflies did not mate in containment in New Zealand (Paynter et al., 2017). Even when they were released and allowed to fly around in a containment greenhouse (5 m long by 2 m wide and 2 m tall), butterflies tended to flutter against the windows trying to escape. Attempts to hand-pair butterflies using a technique developed for North American *Limenitis* species by Platt (1969) were unsuccessful. The inability to mate adults presented a major problem when attempting to establish *L. glorifica* in New Zealand, because weed BCAs must normally be reared through one generation in containment to ensure potential contaminants, such as parasitoids, are eliminated. The first field release in New Zealand was achieved by collecting already-mated adult female butterflies from field sites in Japan in September 2014 (i.e., fall in the northern hemisphere), which were imported into containment in New Zealand. The resulting offspring from these females were then released as their emergence coincided with the New Zealand spring (Paynter et al., 2017). The establishment was achieved at Karangahake Gorge (175.717E 37.417 S) in the Waikato region of New Zealand following a release of 178 adult butterflies in October-December 2014.

In late November 2016, mated adult butterflies were collected from Karangahake Gorge. The initial plan was to obtain offspring from these adults to make another direct field release in New Zealand. However, in early January 2017, several of the freshly emerged adult progeny were opportunistically released in a shade house at the Landcare Research Tamaki Laboratory, in Auckland (174.849E -36.883 S) (shade house dimensions 4 m long by 2 m wide and 4 m tall) containing 10 Japanese honeysuckle plants (potted in planter bags) on which female butterflies could oviposit. The walls of the shade house were made of green plastic mesh and the ceiling was made of opaque white plastic. The temperature and day length in the shade house were similar to ambient outdoor conditions. Butterflies were fed with tissue paper soaked in sports drinks (Pocari Sweat, Otsuka Pharmaceutical Co., Ltd. or Gatorade, The Gatorade Company), which was replenished daily. Butterflies behaved naturally within the shade house, and mating was observed on January 11 and 12, 2017. Approximately 1200 progeny adults and larvae were released at four sites in 2017, and several hundred larvae overwintered on potted honeysuckle plants within the shade house to allow mass-rearing to begin in 2018.

6.4.5.2.2 Brief summary of mass-rearing protocol

In 2018, mass-rearing was conducted at both the Auckland shade house and at the Landcare Research Campus in Lincoln in a greenhouse $4 \times 4 \times 3$ m high, using similar techniques to those employed in 2017. This greenhouse had

whitewashed windows and, in contrast to the situation in containment, butterflies did not beat themselves against the glass trying to escape. This greenhouse was subject to ambient daylength, but temperatures were generally higher than outside and reached 30°C−35°C during the day in summer. In spring, a heater was used to prevent the temperature from falling below ca. 15°C.

Cold tolerance in diapausing *L. glorifica* is associated with a reduction in larval water content and diapause termination involves an increase in water content, as was shown for *Limenitis archippus* (Cramer) (Frankos and Platt, 1976). Potted honeysuckle plants were therefore removed from the Lincoln greenhouse during late fall (May) and left in ambient outdoor conditions until early spring (September) as it was assumed that exposing diapausing larvae to natural rainfall should optimize overwintering success. Shade houses were regularly checked for predators such as spiders and the praying mantis *Miomantis caffra* Saussure, which preyed upon both larvae and adult butterflies.

6.4.5.2.3 Output of mass-rearing, estimated cost, and impact

A total of 57,185 Honshu white admirals were reared between August 2018 and April 2019 which were supplied to local councils for release in batches of 1500−3000 larvae. The total charged for these releases (on a cost-recovery basis) was NZD 92,000 (US$54,529). Mass-rearing enabled many more Honshu white admiral butterflies to be released than would have been possible by redistributing field-collected insects, although it is too early to assess the contribution of mass-rearing to the success of the program.

6.4.5.3 Case study: a leaf-feeding beetle on Tradescentia

The tradescantia leaf beetle *Neolema ogloblini* (Monrós) (Coleoptera: Chrysomelidae) is native to southeastern Brazil and northeastern Argentina (Fowler et al., 2013). Females oviposit on the undersides of *Tradescantia fluminensis* Vell. (Commelinaceae) leaves, sometimes singly but mostly in clusters of 2−5. The eggs hatch after about a week into pale greyish-brown larvae. The larvae accumulate exuviae and excrement which they hold as a protective covering over their backs, presumably to deter predators. The larvae feed and grow through 4 or 5 stadia. Young larvae are gregarious and may be seen forming feeding fronts (Fig. 6.8C). Older larvae feed individually. The pupal cocoons are white and star-shaped and resemble Styrofoam in texture and appearance. New adults (Fig. 6.8D) emerge from cocoons after about 2 weeks. Development from egg to adult takes about 8−10 weeks under ambient summer conditions in a shade house in Auckland (17.5°C−23°C). Beetles are assumed to have three overlapping generations each year in New Zealand and overwinter as adult beetles in the leaf litter.

6.4.5.3.1 Key challenges in designing mass-rearing program

The only major challenge rearing *N. ogloblini* occurred prior to mass-rearing when routine screening of beetles in containment revealed high levels of a gregarine (sporozoan protozoan) gut parasite that appeared to reduce beetle fecundity, longevity and general vigor (Smith et al., 2013). Parasites were eliminated by repeated sub-culturing followed by discarding lines that tested positive for gregarines until a disease-free population could eventually be released from containment (Smith et al., 2013).

6.4.5.3.2 Brief summary of mass-rearing protocol

Rearing protocols were described by van der Walt (2017), who used a similar protocol to that used by Landcare Research in a greenhouse. Seedlings of *T. fluminensis* were grown in 12 cm plastic pots which were then enclosed within fine curtain mesh bags, 50 × 50 cm × 100 cm tall, that were tied shut at the top to enclose the beetles. Around 15−20 beetles were usually released into each bag and then kept in a shade house under ambient conditions similar to those described for *L. glorifica* in Section 6.4.5.2. This process normally resulted in 200−300 beetles emerging per bag in the following 8−10 weeks. Occasionally the numbers reared in a cage were much lower because predators, such as skinks (*Lampropholis delicata* (De Vis)) were inadvertently trapped within a cage (C.J. Winks, personal communication).

6.4.5.3.3 Output of mass-rearing and estimated cost

Between March 2011 and May 2019, a total of 25,050 *N. ogloblini* beetles were reared by Landcare Research which were supplied to councils for release, mainly in batches of 200 to 300 adults. The total cost of rearing these beetles was approximately NZD 300,000 (US$210,420).

6.4.5.3.4 Impact of mass-rearing on establishment and efficacy

Beetles reared by Landcare Research were released at 86 *T. fluminensis*-invaded sites in New Zealand. In addition, instructions on how to rear beetles were provided to stakeholders so that they could conduct their own rearing. Beetles were subsequently reared by both Auckland Council and Wellington City Council. From 2015 to 2017, 770 *N. ogloblini* beetles were released by Wellington City Council at four sites (van der Walt, 2017), and in 2019 approximately 3000 beetles were released at 15 sites by Auckland Council.

Not all release sites have been checked, but the establishment of *N. ogloblini* has been confirmed throughout much of the introduced range of *T. fluminensis* in New Zealand, from Westport on the west coast of the South Island (171.598E 42.763S) to the far north of the North Island (173.532E 35.123S). Establishment and impacts on the target weed have been most impressive in warmer northerly regions and in drier areas away from riverine sites that regularly flood. Recent and ongoing evaluation of *N. ogloblini* impacts indicates that it has already reduced *T. fluminensis* biomass to below the threshold for native forest seedlings to regenerate, allowing forest recovery at several North Island sites (Q. Paynter, unpublished data). The beetle is not yet widespread throughout the remainder of the range of *T. fluminensis* infestation in New Zealand. Mass-rearing is nevertheless winding down because the beetle is now abundant at multiple field sites where it can be collected for redistribution.

6.5 Summary and conclusions

6.5.1 Conclusions from case studies

6.5.1.1 Factors that prompted mass-rearing

The case studies demonstrate two main reasons why mass-rearing of weed BCAs was initiated. The first, and most common (all case studies and most of the examples in Table 6.1) was a need, typically communicated by stakeholders, to release large numbers of the BCA quickly and over a large geographic area (entire states, regions or countries) to obtain widespread establishment, which otherwise might have taken a much longer time. Having a large number of field sites was prioritized over large numbers of released individuals per site, although in most of the case studies the number released per site was substantial (thousands or tens of thousands). Stakeholders likely associated the goal of rapid widespread release with the goal of achieving rapid impact/efficacy, although these need not be linked (Hinz et al., 2020). Historical examples from US and other countries demonstrate the ability of small release populations to rapidly increase, disperse and have major impacts. Although most weeds targeted successfully with biological control are perennials (Coulson, 1977; Pitcairn, 2018), a second key factor, applicable to aquatic weeds and annual or biennial terrestrial weeds, is a need to release large numbers of agents at a vulnerable stage of transition in a short weed lifespan. For example, in South Africa with the advent of the mass-rearing program targeting aquatic weeds, most notably waterhyacinth, management has moved away from a classical biological control approach to an augmentative approach whereby the agents are used as a "green" herbicide (Hill and Coetzee, 2017). This approach allows the re-introduction of BCAs after winter when BCA populations are low, and onto plants missed during herbicide applications (Hill and Coetzee, 2008). On perennial weeds, biocontrol programs using a BCA that rapidly consumes a short-duration and highly vulnerable stage or plant part like immature flower buds (Parrella and Kok, 1979) or that reduces immature weed survival (Havens et al., 2019), may benefit from mass-rearing.

6.5.1.2 Factors conducive to successful mass-rearing

Knowledge of BCA biology: Earlier, we discussed the need for detailed biological information about the BCA and plant-insect interactions to guide the development of mass-rearing protocols. The case studies show that rarely if ever is comprehensive biological information obtained before mass-rearing. However, the most critical aspects that limit and promote BCA development, survival and reproduction are taken into account in protocol development. Observations were as important as empirical data in most of the case studies, as demonstrated in the rearing of *L. glorifica* in New Zealand (make walls of greenhouses and shade houses opaque so that adult butterflies are not attracted to glass walls but rather exhibit normal orientation towards host plants and mates). In the program for UU1 and UU2 on Parkinsonia in Australia, pupae were the focus of rearing and release at remote infestations because of their greater observed tolerance to extreme shipping conditions. In the initial rearing and release of the arundo wasp in the United States, empirical studies to demonstrate the benefits of fertilization and abundant watering were not conducted until after mass-rearing had ended (Moran and Goolsby, 2014; Moran, 2015), but protocols were informed by the literature and anecdotal

observations of poor wasp performance under drought conditions in the native range. Successful rearing of the univoltine houndstongue weevil in Canada demonstrated the feasibility of mass-rearing in the context of difficult BCA life cycles.

Coordination of rearing with known plant-insect field ecology: Mass-rearing was carried out with working or detailed knowledge of ecological factors that could limit establishment, especially seasonal timing of life cycle induction (temperature/day length); the plant phenological stage most suitable for BCA feeding; and types and abundances of potential natural enemies. Rearing was timed to produce BCAs for releases that would best support initial population development. When farms or field sites were used for rearing, as in the Canadian and Australian case studies respectively, steps were taken to reduce biotic interference, and such interactions were also addressed in rearing tunnels in the South African case study.

Involvement of key stakeholders: Stakeholder involvement was essential in all of the case studies to support method development and improvement, operational rearing, and releases. In Australia, the mass-rearing, release and monitoring efforts were achieved through close collaborations between CSIRO, state/territory government collaborators, and multiple regional land management agencies across northern Australia. The success of these collaborations was the result of the following attributes: Identification of multiple nursery sites across northern Australia where regular releases could be made; regular communication between agencies doing mass-rearing and those doing field releases to identify additional sites with Parkinsonia plants in the best possible condition; communication of information outlining the release strategy and its goals to landholders and local weed management/biosecurity officers at field days and through property visits by researchers; and, clarification of the role of biological control within the context of integrated weed management. Partnerships among public agencies and private landholders, nongovernmental organizations and educational institutions can be used to facilitate landscape-scale releases of BCAs for any widely-distributed weed. The waterhyacinth case study in South Africa showed how mass-rearing methods can be specifically designed for implementation by nonscientist users.

6.5.1.3 Costs of mass-rearing

The rearing cost of weed BCAs from past studies ranged between US$1.00 and 3.00/individual for univoltine or other slow-reproducing species (Story et al., 1994, 1996), to less than US$0.50 for one BCA reared on artificial diet (Blossey et al., 2000) and as low as US$0.002 for multivoltine BCAs suitable for outdoor rearing (Harms et al., 2009a,b). Among the case studies above, BCA cost per individual ranged from US$0.06 per water hyacinth planthopper in South Africa to US$8.40 per tradescantia leaf beetle in New Zealand. In most cases, cost figures include distribution as well as rearing. Labor and other costs vary greatly among countries; for example, the annual labor cost in South Africa (1.2 full-time personnel equivalent), is at least ten-fold higher in New Zealand (Q. Paynter, personal observation), so international comparisons are of limited value. Cost per BCA individual may not be the most important factor in evaluating rearing success. For BCAs with long life cycles and relatively low adult rearing yields (example, *C. achates* and *R. donacis* in Table 6.1), the intrinsic value of each individual BCA may be high. Quality control and continuous output monitoring are critical to troubleshooting the system and containing rearing costs. For example, checks for contamination with parasites or pathogens constituted a key quality control factor in the South African and New Zealand case studies.

6.6 Recommendations

6.6.1 Measuring and communicating benefits of mass-rearing

In all the case studies and in the systems profiled in Table 6.1, the BCAs established widespread populations or have shown potential to do so. The global establishment rate for all released weed BCAs is between 60% and 70% (Schwarzländer et al., 2018), which is greater than the percentage of BCAs that have been mass-reared. At least 25% of established agents are expected to have little or no impact (Hinz et al., 2020). Therefore, evidence of establishment alone is not likely to be sufficient to demonstrate the benefits of mass-rearing. The benefits of biological weed control and of rearing can best be determined scientifically in agent efficacy evaluations (Schaffner et al., 2020). Stakeholders will evaluate success in the context of reduced weed densities and spread, but ultimately through perceived improvements in the availability of land and water resources for human use, increased plant biodiversity, and/or economic opportunity. The South African and Australian case studies demonstrate the benefits of closely involving stakeholders in mass-rearing, to increase appreciation of the benefits of mass-release of efficacious BCAs. In all cases in which BCA populations reach an impacting level, a clear tangible result can be communicated to funding agencies and individuals.

Mass-rearing operations are temporary by design. In performing efficacy evaluations, scientists should take care to not leave stakeholders behind but rather continue to inform and engage them, to benefit potential future projects.

6.6.2 Keeping mass-rearing at the forefront of implementation

The history of biological weed control suggests that mass-rearing has largely been viewed as a secondary or "last resort" measure when other implementation methods fail. Mass-rearing should in fact be considered for every new weed BCA being released. To gain stakeholder support, "proof of concept" (a scalable rearing protocol leading to BCA establishment at a few sites) is likely to be required. Cost-benefit analyses can be used to guide decision-making not only prior to initiation of new biological weed control projects or new searches for BCAs (Deveze, 2004; Seawright et al., 2009) but also when deciding whether to mass-rear one or more recently-permitted BCA(s). Retrospective assessments (van Wilgen et al., 2020) can be used to reinforce program benefits. Rapid establishment of BCAs over a large area is the most desirable result. The relationship between BCA establishment and impact on the weed (i.e., to create an actual benefit to the environment and to stakeholders) is not predictable, but in all of the case studies (except *L. glorifica* in New Zealand for which evaluations are ongoing), both establishment and impact have been observed. In the 2000s the number of candidate agents permitted for release declined (Schwarzländer et al., 2018), due in part to increased selectivity among practitioners of BCAs prioritized for characterization and release, based on their predicted efficacy. Running approximately counter to this trend is a significant increase in the number of agents being mass-reared (except for prickly-pear, Table 6.1 and the case studies date from the 1990s to present). A greater emphasis on increasing the likelihood of success for each released BCA may be responsible.

6.6.3 Frontiers in mass-rearing of weed biological control agents

Other chapters in this book cover empirical and mathematical methods to assess the quality of mass-reared arthropods, and these could be applied to weed BCAs. The ability of mass-reared BCAs to adapt to suboptimal host plants and climatic conditions in comparison to BCAs that were not mass-reared (Müller-Schärer and Schaffner, 2008) is an unexplored topic and has relevance to predicting outcomes when BCA populations are dispersing at regional or continental scales. As weeds expand their ranges, laboratory-based programs breeding for adaptation in BCAs to environments that differ from their native range, for example, with semitemperate rather than subtropical climates (Harms et al., 2021) or with differing photoperiod regimes (Bean et al., 2007) would benefit from mass-rearing protocols. Finally, the development of gene drives for insects (Brossard et al., 2019) may offer the possibility to reversibly field-test BCAs for which there are concerns about host range, as a sterility gene would preclude reproduction and spread; mass-rearing would be essential for these studies.

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Chapter 7

Mass production of predatory mites: state of the art and future challenges

Dominiek Vangansbeke, Marcus V.A. Duarte, Apostolos Pekas, Felix Wäckers and Karel Bolckmans
Biobest N.V., R&D Department, Ilse Velden 18, Westerlo, Belgium

Most mites are minuscule, barely perceptible to even the sharpest eyes, but size is a poor guide to importance.
David. E. Walter & Heather C. Proctor

7.1 Introduction

7.1.1 Mites and their importance as biocontrol agents

Mites are among the smallest arthropods thriving on our planet. They are usually smaller than 1 mm leaving them unnoticed by the untrained eye. However, mites do have a profound impact on various aspects of human life. Mites cause damage to crop plants (e.g., *Aculops* spp., *Tetranychus* spp.) or poultry stocks [e.g., *Dermanyssus gallinae* (De Geer)] on which we feed. Mites can burrow human and animal skin [*Sarcoptes scabiei* De Geer (Arlian, 1989)] or feed on dead skin cells [*Demodex* sp. (Grice and Segre, 2011)]. Some mite species are the causal agent of allergies in house dust [*Dermatophagoides* sp. (Plattsmills and Chapman, 1987)]. In apiculture, mites are found inside the tracheae of bees [*Acarapis woodi* (Rennie), (Sammataro et al., 2000)] or infesting the brood and transmitting viruses in honeybees [*Varroa destructor* (Anderson and Trueman), (Ramsey et al., 2019)].

However, mites can be useful to us as well. Besides acting as nutrient recyclers in decomposing soil and seasoning our cheeses (e.g., *Acarus siro* L. and *Tyrolichus casei* Oudemans), mites can be employed as predators to protect crops from damaging pests.

Predatory mites are the most important commercially available group of beneficial arthropods used in augmentative biological control programs worldwide (Knapp et al., 2018; van Lenteren, 2012). In protected cultivation, predatory mites successfully control insect pests, such as several thrips and whitefly species, and mite pests, such as broad mites and spider mites (Gerson and Weintraub, 2007). More specifically, predatory mites from the family Phytoseiidae account for four out of ten of the most important commercially available arthropod biological control agents based on the turnover of biocontrol companies (Knapp et al., 2018). Together, *Amblyseius swirskii* (Athias-Henriot), *Phytoseiulus persimilis* Athias-Henriot, *Neoseiulus californicus* (McGregor), and *Neoseiulus cucumeris* (Oudemans) comprise 60% of the total market for arthropod natural enemies (Knapp et al., 2018) (Table 7.1).

7.1.2 Brief historical overview

In the late 1960s, the phytoseiid *P. persimilis* was one of the first augmentative biocontrol agents to become commercially available (van Lenteren, 2012). This predator is specialized in preying upon spider mites and is sometimes referred to as the "acaricide on legs" because of its effectiveness in controlling these pests (Helle and Sabelis, 1985; Hoy, 2009). Nowadays, *P. persimilis* is still a key component in the biological control of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). Later, in the 1980s, *Neoseiulus barkeri* (=*mckenzie*) Hughes and *N. cucumeris* proved successful in the biological control of *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Ramakers and Van Lieburg, 1982). Soon after, the invasion of the western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) in the Netherlands, further triggered the research interest in phytoseiid predatory mites

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TABLE 7.1 Top 10 of most important arthropod biological control agents by turnover (van Lenteren 2012; Knapp et al., 2018).

1	***Amblyseius swirskii***
2	***Phytoseiulus persimilis***
3	***Neoseiulus californicus***
4	*Macrolophus pygmaeus*
5	*Encarsia formosa*
6	*Orius laevigatus*
7	*Nesidiocoris tenuis*
8	***Neoseiulus cucumeris***
9	*Eretmocerus eremicus*
10	*Aphidius colemani*

Note: Phytoseiid mites in bold letters.

(Messelink et al., 2006). The potential of phytoseiids for the control of whiteflies was reported about 50 years ago (e.g., El-Badry, 1967; Swirski et al., 1967; Teich, 1966). It was not until the work of Nomikou et al., which triggered the biocontrol companies' interest in phytoseiids for the control of the whitefly pests, such as *Bemisia tabaci* (Gennadium) and *Trialeurodes vaporariorum* Westwood (Bolckmans et al., 2005; Medd and GreatRex, 2014; Nomikou et al., 2001). The most promising predatory mite for the control of whiteflies turned out to be *A. swirskii* (Nomikou et al., 2001). Furthermore, in 2005, Greenpeace Germany reported serious exceedances of the maximum residue level of a range of pesticides on sweet pepper fruits originating from Spanish greenhouses. Later, the presence of illegal pesticides on fruits and vegetables was reported. As a result, the interest and need for alternatives to chemical control, such as biocontrol-based integrated pest management (IPM), sharply increased under pressure from the European supermarkets who imposed very strict extra-legal requirements on their fruits and vegetables in order to prevent any future food scandals. At the same time, *A. swirskii* became commercially available and showed promising results for whitefly and thrips control under greenhouse trial conditions in many crops (Calvo et al., 2012). Stimulated by the reasons mentioned above, the vast majority of the growers in Almeria (Southeastern Spain) switched to the use of augmentative biocontrol within an IPM framework, with phytoseiid predatory mites as the mainpillar of the pest control strategy (Calvo et al., 2015 and references therein). Today *A. swirskii* is widely used for biological control of thrips and whiteflies in greenhouse vegetables, berries and ornamentals.

A crucial parameter contributing to this successful adoption was the possibility to mass-produce predatory mite on factitious prey mites, which in turn can be reared on cheap and simple food sources (Calvo et al., 2015; Ramakers and Van Lieburg, 1982). Without an affordable, efficient and reliable mass-rearing system, promising (acarine) biological control agents will never be employed in biocontrol-based IPM programs.

This chapter documents the evolution and current state of the different predatory mite production systems, the associated pros and cons and the challenges to further expanding the scope of acarine biocontrol agents inside and outside greenhouse-grown crops. Seen their commercial importance, the focus will be on phytoseiid predatory mites. However, we will briefly discuss other predatory mite families that are commercially available or have the potential to become so.

7.2 Phytoseiidae

7.2.1 Lifestyles of phytoseiid predatory mites

Phytoseiid predatory mites feed on a diverse range of food sources. Mites like *P. persimilis* are highly specialized in feeding on spider mites (Helle and Sabelis, 1985), whereas others, like *Euseius* sp., have a strong preference for pollen (Adar et al., 2014; Broufas and Koveos, 2000). Some phytoseiids, like *Euseius scutalis* (Athias-Henriot) and *Iphiseius degenerans* (Berlese), need to feed upon plant material to absorb water (Adar et al., 2012), whereas others can maintain

their water balance if the ambient relative humidity is above a certain threshold level (Gaede, 1992) by absorbing ambient water. Such diet restrictions dictate the mass-rearing possibilities for the predators.

The varying degree of specialization toward feeding and reproducing on tetranychid mites motivated McMurtry and Croft (1997) to classify the different lifestyles of phytoseiids. Depending on their feeding habitats, phytoseiids were categorized into four groups with different levels of specialization on spider mite prey. Later, this categorization was further subdivided (see McMurtry et al. (2013), Table 7.2), including prey mite specificity and substrate as parameters. A summary of the lifestyles of the most important (commercially available) phytoseiid predatory mite species is presented in Table 7.2. As will become clearer later in this chapter, knowledge of the specific lifestyle is essential in developing a rearing system. For details, we refer to the papers of McMurtry and Croft (1997) and McMurtry et al. (2013).

Type I predators are specialized predators of tetranychid mites having a dense, complicated webbing, including predatory mites from the genus *Phytoseiulus*. Similar to Type I predators, Type II predators are also associated with spider mites; however, they show a broader prey range on which they can feed and reproduce. For example, *N. californicus* is an important commercially available spider mite predator, which can also feed and reproduce on astigmatid mites like *Lepidoglyphus destructor* (Schrank) and *Glycyphagus domesticus* (de Geer) (Castagnoli et al., 2006) or other pest mites like the tarsonemid *Polyphagotarsonemus latus* (Banks) (Peña and Osborne, 1996). Moreover, *N. californicus* can also feed and reproduce on plant-derived foods, such as pollen (Khanamani et al., 2017; Yuan et al., 2020). The Type II predator group includes species from the genera *Neoseiulus*, *Galendromus*, and to a lesser extent, species from the genus *Typhlodromus*.

More generalist predatory mites, for which web-forming tetranychids are usually an unsuitable prey, belong to Type III. Nearly all phytoseiid genera, except for *Phytoseiulus*, *Galendromus* and *Euseius*, have species in this group. The diet of Type III predators includes other mite species, insect prey, plant-provided pollen and extrafloral nectar. Usually, oviposition on those food sources is higher than that on tetranychid prey. A possible disadvantage of mass-rearing of Type II predators is the more frequent occurrence of cannibalism and interspecific predation.

Specialized pollen feeders belong to the Type IV group, which comprises mainly species from the genus *Euseius*. In this group, phytoseiids are usually polyphagous. However, the reproductive performance of pollen is superior compared to that when fed on other prey food. Species of *Euseius* can also need to extract water from plant tissue. The pollen preference is also reflected in a typical cheliceral shape, possibly adapted for pollen and plant-feeding (Adar et al., 2012; Flechtmann and McMurtry, 1992).

TABLE 7.2 Feeding habits of Phytoseiidae according to McMurtry et al. (2013).

Type	Feeding habit	Example commercially available species
Type I	Specialized mite predators	
	(a) Specialized predators of *Tetranychus* (Tetranychidae) (b) Specialized predators of web-nest producing mites (Tetranychidae) (c) Specialized predators of tydeoids (Tydeoidea)	(a) *Phytoseiulus persimilis*[a], *Phytoseiulus macropilis*[a] (b) *Typhlodromus* (*Anthoseius*) *bumbusae* (c) *Typhlodromina eharai*, *Proprioseiopsis* sp.
Type II	Selective predators of Tetranychid mites	*Neoseiulus californicus*[a], *Neoseiulus fallacis*[a], *Neoseiulus longispinosus*
Type III	Generalist predators	
	(a) Generalists living on pubescent leaves (b) Generalists living on glabrous leaves (c) Generalists living in confined space of dicotyledonous plants (d) Generalist living in confined spaces of monocotyledonous plants (e) Generalists from soil/litter habits	(a) *Kampimodromus aberrans*, *Galendromus occidentalis*, *Typhlodromus (Typhlodromus) pyri*[a], *Phytoseius finitimus* (b) *Amblyseius swirskii*[a], *Amblydromalus limonicus*[a], *Transeius montdorensis*[a], *Amblyseius andersoni*[a] (c) *Amblyseius herbicolus*, *Iphiseius degenerans*[a], (d) *Neoseiulus baraki*, *Neoseiulus paspalivorus* (e) *Neoseiulus barkeri*[a]
Type IV	Specialized pollen feeders/generalist predators	*Euseius* sp. (*E. gallicus*[a], *E. stipulatus*, *E. scutalis*)

[a]*Commercially available (or have been commercially available).*

7.2.2 Mass-rearing systems for phytoseiid predatory mites

Bolckmans (2007) proposed a classification of mass-rearing systems for phytoseiid mites. The classification shows how mass-rearing systems have evolved to an increasingly higher level of controllability by eliminating trophic levels (evolution from tritrophic over bitrophic to eventually monotrophic rearing systems) and moving from production in greenhouses to production in climate rooms, allowing a better control of climatic conditions and contaminants (Table 7.3).

7.3 System 1: both tetranychid prey mites and predatory mites are produced on plants in greenhouses

The most "natural" and oldest rearing method to produce phytoseiid mites consists of a tritrophic system including a host plant, a mite prey and the mite predator. This system is usually employed to produce Type I specialized tetranychid predators, such as *Phytoseiulus* spp. McMurtry et al. (2013) (Table 7.2). The high level of effectivity of this highly specialized spider mite predator poses important limitations when it comes to its mass production, as spider mites are the only accepted food source (Hoy, 2009). The spider mite prey, in turn, can only be produced efficiently on plant material as well. All three trophic levels need to be managed, requiring high input of time, space, energy, and labor (Fournier et al., 1985).

All steps of this "batch-wise" rearing process occur in a greenhouse environment and require three separate stages: first, growing bean plants, then the rearing of spider mites, and finally, the rearing of the predatory mite (Scopes, 1968). Bean plants (mostly *Phaseolus vulgaris*, sometimes *Vicia faba*) are sown in clean, pest-free greenhouse compartments. Upon the emergence of the first true leaves, they are infested with spider mites. When the bean plants reach a certain level of infestation, predatory mites are released. Predatory mites are allowed to build up in population sizes to a level where nearly all spider mite prey is consumed (Fig. 7.1). Next, predatory mites can be collected by either harvesting leaves with predators (Morrison and King, 1977) or by allowing the predators to move away from the leaf material. In the former case, leaves containing the predatory mites are collected in (preferably paper) bags for transport to the end-user. When spider mite densities are being depleted, *P. persimilis* will start dispersing (Pels and Sabelis, 1999; van Baalen and Sabelis, 1995). To collect these mites, producers exploit the negative geotropism and positive phototropism of the predatory mites. When dispersing, mites tend to move upwards and toward the light, thereby allowing collection (Morales-Ramos et al., 2012).

This tri-trophic rearing system is vulnerable to many factors that can seriously compromise the efficiency and eventually the reliability of the rearing system. Contamination with secondary pests, such as the western flower thrips *F. occidentalis* and leafminers (*Liriomyza* spp.) can be a serious problem if insufficient precautionary measures are taken. This omnivorous thrips species not only feeds on *T. urticae* eggs (Trichilo and Leigh, 1986), but also kills phytoseiid eggs (Janssen et al., 2002), including *P. persimilis* eggs (Walzer and Schausberger, 2009). Besides secondary plant pests, secondary predators, which compete with *P. persimilis* for *T. urticae* food, may also invade the spider mite populations. Other predators, such as other predatory mite species (Ho and Chen, 1992), predatory coleopterans (*Stethorus* sp. (Coccinellidae), *Oligota* sp. (Staphylinidae), and gall midges (*Feltiella* sp. (Cecidomyiidae)) may enter the rearing greenhouse, thereby disturbing the growth of the spider mite colonies. Finally, the presence of contaminants in the end material is highly undesirable and can make the end-product unsuitable for sales. Short production cycles of the bean plants (Scriven and McMurtry, 1971), stringent hygiene, pest exclusion methods (insect-proof netting) and infesting with spider mites and predatory mites from a pure mother culture help to prevent contaminations. Entomopathogenic fungi, such as the obligate spider mite pathogen *Neozygites* sp. (see later), may infect spider mites leading to the destruction of the rearing populations (Delalibera et al., 2004; Duarte et al., 2009; Wekesa et al., 2007). Up to date, the majority of the biocontrol companies still use this rearing system for *Phytoseiulus* spp. as it yields high numbers of predators and reliable alternative rearing systems are lacking. Besides *Phytoseiulus* species, some biocontrol producers also produce type II selective predators, such as *N. californicus* and *N. fallacis*, in this way.

7.4 System 2: tetranychid prey mites are reared on plants in greenhouses. The predator is reared in climate rooms on detached leaves with prey mites

The next step that dealt with some of the challenges of the traditional plant system was proposed by Fournier et al. (1985). Whereas tetranychid prey mites are still produced on bean plants as was described for rearing System 1, the predatory mites are produced in climate rooms on detached bean leaves that are heavily infested with spider mites. Heavily infested bean leaves are collected from the plants and placed in rearing cylinders with a nylon mesh at the

TABLE 7.3 Different types of rearing systems for predatory mites (Bolckmans 2007).

System	Type of predatory mite (McMurtry and Croft, 1997)	Food source	Food source production	Predator production	Labor	Cost	Space	Species of predatory mites
1	Spider mite predator (Types I and II)	Tetranychid spider mites on plants	In greenhouses on plants	In greenhouses on plants	++++	+++	++++	*P. persimilis*, *N. californicus*, *N. fallacis*
2	Spider mite predator (Types I and II)	Tetranychid spider mites on detached bean leaves	In greenhouses on plants	In climate rooms	+++	+++	+++	*P. persimilis*, *N. californicus*, *N. fallacis*
3	Spider mite predator (Types I and II)	Tetranychid spider mites isolated from any plant material	In greenhouse on plants	In climate rooms	+++	++++	+++	*P. persimilis*, *N. californicus*, *N. fallacis*
4	Generalist predator (Types II and III)	Factitious prey reared on a simple food source such as yeast	In climate rooms	In climate rooms	++	++	++	*N. californicus*, *N. fallacis*, *A. swirskii*, *N. cucumeris*, *A. limonicus*, *T. montdorensis*, *A. andersoni*
5	Generalist predator (Types III and IV)	Pollen	In greenhouse on plants	In greenhouse on plants	+++	+++	+++	*A. limonicus*, *E. gallicus*, *I. degenerans*
6	Generalist predator (Types II and III)	artificial diet	in laboratory	In climate rooms	+	+	+	*N. californicus*, *N. fallacis*, *A. swirskii*, *N. cucumeris*, *A. limonicus*, *T. montdorensis*

FIGURE 7.1 Production facility of *Phytoseiulus persimilis* on bean plants heavily infested with *Tetranychus urticae*.

bottom. A certain number of predatory mites are being released and are allowed to feed and reproduce. The proportion of predator/prey can be monitored by either observing the leaf material or by a glass collection tube on the top of the rearing cage. The presence of *P. persimilis* in those tubes is a suitable proxy for prey depletion due to the negative geotropic behavior of *P. persimilis*. At this point, a new cage, containing new leaves infested with *T. urticae*, is placed on top of the cage that contained the collection tube. Then, the oldest cages can be removed as those will no longer contain either predators or prey.

For collection, the mites can be siphoned by using an aspirator. However, for large greenhouse introductions, this is a labor-intensive and time-consuming operation. To facilitate subsequent introduction in the crop, bran carrier can be added to the cages. While stirring, the bran carrier will collect the predatory mites and fall to the bottom of the cage (Fournier et al., 1985).

The major advantage of this system is that predator production can take place in a controlled environment. Humidity and temperature can be set for optimal predatory mite growth while at the same time further limiting the risk of contamination. Collection in bran material also allows a fast and reliable estimation of the predatory mites' density in the end material. By counting the number of mites inside a subsample of the bran, the total number and density of the predatory mites can be easily estimated. Finally, the reduced space and labor requirements were mentioned as the major advantages of this system (Fournier et al., 1985).

Phytoseiid predatory mites require a moist environment to optimize their water balance and developmental and reproductive performance (Gaede, 1992; Stenseth, 1979). As a result of the required high humidity conditions, leaf material in this system will tend to rot and grow molds (Fournier et al., 1985). To avoid this, eliminating leaf material by separating spider mites from the leaf material is a further step toward optimization.

7.5 System 3: tetranychid prey mites are reared on plants in greenhouses. The predator is reared in a climate room on pure prey mite stages

In a further step to eliminate plant material and the associated mold growth (Fournier et al., 1985), spider mites are separated from the plant material before being offered to the predator.

McMurtry and Scriven (1966) reported one of the first rearing systems using tetranychid prey mites. Pacific spider mites, *Tetranychus pacificus* McGregor, were grown on oranges, as described by Scriven and Fleschner (1960). Spider mites were brushed off from the oranges and provided to *Phytoseiulus* spp., which were reared on an artificial plastic substrate. This method reduces the problems associated with plant material. However, fruit availability throughout the year and the cost of the fruits are a limitation of this method. To overcome this problem, Scriven and McMurtry (1971) proposed another system using bush lima bean, *Phaseolus limensis* L., which is sown in vermiculite instead of potting soil. One-week-old lima bean seedlings then are infested with 0.24 g of *T. pacificus* eggs per 100 lima bean plants. Two weeks later, a five-fold increase in mite weight can be harvested for predator feeding. In this case, tetranychid mites are separated from the leaf material by a leaf washer/mite separator. Harvested bean leaves are placed inside the washer, which is a resin-coated plywood box with a volume of 205 liters. The washer is filled with water and 2 liters of bleach

solution (5% NaClO) and a little liquid detergent. A small stream of water added to the washer creates an overflow, which will collect the spider mites into the mite separator. This mite separator allows separation of females, immatures and males and finally eggs and larvae. Subsequently, different mite stages can be washed off, dried and cold-stored before use. No less than 12.9 g *T. pacificus*/1000 plants could be harvested using this method. A disadvantage is the (undetermined) mortality of spider mites caused by the washing procedure.

A further step was proposed by Shih (2001), where hydroponic lima beans (*Phaseolus lunatus* L.) were grown in a polyethylene basket. A major advantage, of growing the beans hydroponically is that plants can be kept upside down in a washing/rinsing machine. Spider mites were provided to the predatory mite on bakelite sheets using a micro-feeding gun. This method was developed for *Neoseiulus longispinosus* (Womersley), a type II selective spider mite predator (McMurtry et al., 2013).

Another method of mass-producing *Phytoseiulus* sp. describes a system where 15-day-old broad bean (*V. faba* L.) plants, which were infested with 100−200 *T. urticae* (Scopes, 1968). When so-called "ropes" of dispersing spider mites start to form, they are collected and cold-stored before use. Such collections can be done for up to 4 consecutive days. Then, the remaining leaves are harvested, and mites are separated from the leaves and debris by washing them with water through sieves. Washed out mites are collected and dried on filter papers. Such filter papers can be stored up to a month before offering them to the predatory mites. Similarly, Theaker and Tonks (1977) cultured the spider mites first on *P. vulgaris*. Spider mites were separated from the leaf material using a "mite-brushing device."

Rather than a two-dimensional rearing system of the predatory mites on plastic plates or bakelite sheets, which requires a lot of space and makes it difficult to contain the mites, predatory mites can be efficiently mass-reared in containers with a suitable carrier material, such as saw dust or corn cob grits, and provided at regular intervals with pure spider mite eggs. Such a three-dimensional rearing system requires much less space and facilitates containment of the predatory mites while excluding potential contaminants.

7.6 System 4: predatory mites are grown on factitious food sources

7.6.1 Factitious prey mites

A giant leap forward in the rearing efficiency of generalist predatory mites was the development of a rearing system using factitious prey mites. In 1960, Burnett (1960) showed the potential of using the astigmatid mites *Tyrophagus castellanii* (Hirst) and *A. siro* for rearing the predatory mites *Cheyletus eruditus* (Schrank) (Cheyletidae) and *Melichares dentriticus* (Berlese) (Blattisocidae). The first report on using astigmatid prey mites for mass-producing phytoseiids was published by Schliesske (1981). This paper, however, was heavily inspired by the work of Pierre Ramakers and colleagues in the Netherlands. In 1982, Ramakers and Van Lieburg (1982) reported the potential of using astigmatids for commercial predatory mite production potential of using *Acarus* spp. as an alternative food source for *N. cucumeris* and *N. barkeri*. Mass-rearing of *N. cucumeris* and *N. barkeri* on their target prey, *Thrips tabaci*, was not possible as the production of thrips was not efficient enough (Ramakers and Van Lieburg, 1982). A huge improvement was made by offering the predatory mites *Acarus farris* (Oudemans) (Acari: Acaridae) as prey, which could be produced at high densities on inexpensive food sources, such as sterilized bran. This method showed potential for the generalist predatory mites *N. cucumeris*, *N. barkeri*, and *A. andersoni*. Later, also the house dust mite *Dermatophagoides farinae* Hughes (Acari: Pyroglyphidae) was found to be a suitable food source for *N. californicus* (Castagnoli et al., 1999). This research demonstrated that also Type II selective spider mite predators could be produced on (cheaper) food sources other than tetranychid spider mites. A breakthrough was the discovery of the potential of the dried fruit mite *Carpoglyphus lactis* (L.) for a wide range of phytoseiids (Bolckmans and van Houten, 2006b). This astigmatid prey mite showed to be an excellent food source for phytoseiids (Ji et al., 2015; Nguyen et al., 2013; Vangansbeke et al., 2014b) and was the foundation of the commercial success of *A. swirskii* (Calvo et al., 2015). However, *Carpoglyphus lactis* turned out not to be suitable for mass-rearing *N. californicus*. In follow-up studies, *Lepidoglyphus destructor* and *Glycyphagus domesticus* (Acari: Glycyphagidae) proved to be a highly suitable food source for mass-rearing *N. californicus* (Bolckmans et al., 2007; Castagnoli et al., 2006). Since then, many Astigmata have been collected, tested and used for a range of phytoseiids (see Table 7.4 for examples). The patents on the use of Caropglyphidae (Bolckmans and van Houten 2006b) and Glycyphagidae (Bolckmans et al., 2007) for mass-rearing phytoseiid predatory mites subsequently triggered a patenting spree on mass-rearing technologies and suitable prey mite species for predatory mites in the biocontrol industry (Fig. 7.2).

Besides being a suitable food source for the predators, most factitious prey mites are fungivorous and play an important role in controlling fungal growth on the rearing medium. Production of phytoseiids typically requires a high ambient

TABLE 7.4 Exemplary studies testing prey mites for the production of predatory mites.

Cohort	Family	Prey mite species	References	Predatory mites	Findings
Astigmata	Carpoglyphidae	*Carpoglyphus lactis L.*	Bolckmans and van Houten (2006a)	*Amblyseius swirskii* (Athias-Henriot) and *Neoseiulus cucumeris* (Oudemans) (Phytoseiidae)	Both predatory mite species were able to reproduce on *C. lactis*.
			Bolckmans and van Houten (2006b)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Predatory mite can reproduce on *C. lactis*.
			Fidgett and Stinson (2008)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Mass production of the predatory mite on *C. lactis* yielded lower results as compared to using *T. entomophagus*.
			Grosman et al. (2011)	*Macrocheles robustulus* (Berlese) (Macrochelidae)	Laboratory colonies of *M. robustulus* were maintained on *C. lactis*.
			Nguyen et al. (2013)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Predatory mites showed higher or equal intrinsic rate of increase (r_m) on *C. lactis* as compared to pollen and artificial diets.
			Vangansbeke et al. (2014a)	*Amblydromalus limonicus* Garman and McGregor (Phytoseiidae)	Predatory mites showed higher or equal intrinsic rate of increase (r_m) on *C. lactis* as compared to natural prey and factitious foods.
			Ji et al. (2015)	*A. swirskii* (Athias-Henriot), *Amblyseius eharai* Amitai and Swirski and *N. cucumeris* (Oudemans) (Phytoseiidae)	*C. lactis* was a suitable food source for all three predatory mites species tested. The highest intrinsic rate of increase (r_m) was obtained for *A. eharai*, followed by *A. swirskii* and finally *N. cucumeris*.
			Tabic et al. (2019)	*Phytoseiulus persimilis* Athias-Henriot (Phytoseiidae)	A selected strain of the predatory mite was able to reproduce for multiple generations on frozen prey mite.
	Glycyphagidae	*Lepidoglyphus destructor* (Schrank)	Barker (1991)	*Cheyletus eruditus* (Schrank) (Cheyletidae)	*C. eruditus* can survive, develop and reproduce when feeding on *L. destructor*
			Bolckmans et al. (2007)	*Neoseiulus fallacis* (Garman) *Neoseiulus californicus* (Phytoseiidae)	Both predatory mite species were able to reproduce on *L. destructor*. *N. californicus* was able to maintain a high intrinsic rate of increase on *L. destructor* for multiple generations.
			Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	Predatory mite showed highest egg production as compared to that on *T. casei*, *T. longior*, *G. domesticus*, *A. siro* and *D. farinae*. Laboratory cultures could be maintained easily for >2years.
			Simoni et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	High intrinsic rates of increase were obtained for *N. californicus* when reared for multiple generations on *L. destructor*.
			Cebolla et al. (2009)	*Cheyletus malaccensis* Oudemans (Cheyletidae)	Intermediate intrinsic rate of increase was obtained on *L. destructor*, whereas a higher r_m was observed when fed *T. putrescentiae*
		Glycyphagus domesticus (De Geer)	Barker (1969)	*Hypoaspis aculeifer* (now *Gaelolaelaps aculeifer*) Canestrini (Laelapidae)	*G. domesticus* resulted in a higher intrinsic rate of increase of compared to feeding on *T. putrescentiae*.

			Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	Predatory mite shows an equally high egg production as compared to L. destructor. Escape rate of *N. californicus* from the rearing arenas was higher as compared to *L. destructor*.
	Pyroglyphidae	*Dermatophagoides farina* Hughes	Castagnoli et al. (1999)	*N. californicus* (McGregor) (Phytoseiidae)	Predatory mite showed a relatively low intrinsic rate of increase on *D. farinae* as compared to its natural prey, *T. urticae*. *D. farinae* is however considered as potential food source for mass-producing *N. californicus*
			Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	Predatory mite showed relatively low egg production as compared to that on *G. domesticus* and *L. destructor*.
		Dermatophagoides pteronyssinus (Trouessart)	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	High egg production for *I. zuluagai*, intermediate egg production for *N. barkeri* and *N. californicus* and low egg production for *E. concordis*.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
		Hughesiella africana (Hughes)	Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
	Chortoglyphidae	*Chortoglyphus arcuatus* (Troupeau)	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition as compared to other tested astigmatid prey mites.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
	Echimyopodidae	*Blomia tropicalis* Bronswijk	Barbosa and de Moraes (2015)	*N. californicus* (McGregor) (Phytoseiidae)	Equally high oviposition rate as on *A. lukoschusi*.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
	Aeroglyphidae	*Austroglycyphagus lukoschusi (Fain)*	Barbosa and de Moraes (2015)	*N. californicus* (McGregor) (Phytoseiidae)	Equally high oviposition rate as *B. tropicalis*.
	Acaridae	*Tyrophagus putrescentiae* (Schrank)	Barker (1969)	*Hypoaspis aculeifer* (now *Gaelolaelaps aculeifer*) Canestrini (Laelapidae)	*T. putrescentiae* resulted in a lower intrinsic rate of increase of compared to feeding on *G. domesticus*.
			Bolckmans and van Houten (2006b)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Predatory mites can reproduce on *T. putrescentiae*.
			Cebolla et al. (2009)	*Cheyletus malaccensis* Oudemans (Cheyletidae)	Highest intrinsic rate of increase were obtained when fed *T. putrescentiae*, higher than those on *A. siro*, *L. destructor*, *A. ovatus* or *C. redickorzi*.
			Sarwar et al. (2010)	*Neoseiulus pseudolongispinosus* (Xin, Liang and Ke) (Phytoseiidae)	

(*Continued*)

TABLE 7.4 (Continued)

Cohort	Family	Prey mite species	References	Predatory mites	Findings
					Prey mites were found to be suitable for the predatory mite when the latter was grown on flour of wheat, but to a lesser extent on flour of soybean and maize.
			Grosman et al. (2011)	*Hypoaspis aculeifer* (now *Gaelolaelaps aculeifer*) Canestrini (Laelapidae)	Laboratory colonies of *H. aculeifer* were maintained on *T. putrescentiae*.
			Huang et al. (2013)	*Neoseiulus barkeri* Hughes (Phytoseiidae)	Population growth of the predatory mites on the prey mites was significantly improved by adding yeast powder, sugar and glucose.
			Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition as compared to other tested astigmatid prey mites.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition rate as compared to other astigmatid prey mites.
			Cakmak and da Silva (2018)	*Hypoaspis larvicolus* Joharchi and Halliday (Acari: Laelapidae)	Predatory mite showed lowest population growth on *T. putrescentiae* as compared to the *A. siro* and *C. lactis*.
			Pirayeshfar et al. (2020)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Oviposition rate of predatory mite on frozen prey mite larvae was equally high as compared to *Typha* sp. pollen.
		Tyrophagus longior Gervais	Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	*T. longior* was not suitable as food source as juveniles could not develop to adulthood and egg production was absent.
		Tyrophagus similis Volgin	Saito and Takaku (2013)	*Hypoaspis yamauchii* Ishiwaka (Laelapidae)	The predatory mite fed on all nonegg stages of the prey mite.
		Tyrophagus curvipenis Fain and Fauvel	Li et al. (2021)	*N. cucumeris* (Oudemans) (Phytoseiidae)	Prey mite was found to be an excellent food source.
		Mycetoglyphus fungivorus Oudemans	Momen et al. (2020)	*Neoseiulus barkeri* (Hughes) (Phytoseiidae)	Prey mite could support *N. barkeri* for 6 generations, albeit with lower reproduction as compared to *E. kuehniella* eggs.
		Thyreophagus entomophagus (Laboulbene)	Fidgett and Stinson (2008)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Mass production of the predatory mite on *T. entomophagus* yielded better results as compared to using *C. lactis*.
		Thyreophagus cracentiseta Barbosa, O'Connor & de Moraes	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Feeding on this prey mite resulted in highest oviposition rate for *N. barkeri*, and intermediate oviposition rates for the other predaory mites as compared to other astigmatids tested.

			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	*A. tamatavensis* showed the highest daily oviposition rate on this prey mite as compared to other astigmatid prey mites. Intermediate oviposition rate for *N. anonymus*.
		Aeroglyphus robustus (Banks)	Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
		Aleuroglyphus ovatus Troupeau	Xia et al. (2012)	*Neoseiulus barkeri* Hughes (Phytoseiidae)	Prey mite was found the be an excellent food source for the predatory mite.
			Cebolla et al. (2009)	*Cheyletus malaccensis* Oudemans (Cheyletidae)	Intrinsic rates of increase were lowest when fed *A. ovatus* as compared to *A. siro*, *L. destructor*, *C. redickorzevi* i or *T. putrescentiae*.
			Guichou et al. (2015)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Feeding on eggs of the prey mite increased the performance of the predatory mite.
			Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition as compared to other tested astigmatid prey mites.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
		Cosmoglyphus oudemansi (Zachvatkin)	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition as compared to other tested astigmatid prey mites.
		Caloglyphus redickorzevi (Zachvatkin)	Cebolla et al. (2009)	*Cheyletus malaccensis* Oudemans (Cheyletidae)	Intermediate intrinsic rate of increase was obtained on *C. redickorzevi*, whereas a higher r_m was observed when fed *T. putrescentiae*.
		Caloglyphus rodriguez Samsinak	Fouly and Abdel-Baky (2005)	*Cosmolaelaps qassimensis* Fouly and Al-Rehyiani (Laelapidae)	Predatory mite showed a lower total number of eggs as compared to *T. putrescentiae*. Intrinsic rate of increase on *C. rodriguez* was found to be higher as compared to egg masses of the root-knot nematode *Meliodogyne incognita* Chitwood.
		Sancassania berlesei (Michael)	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition as compared to other tested astigmatid prey mites.
		Tyrolichus casei Oudemans	Rasmy et al. (1987)	*Agistemus exsertus* Gonzalez (Stigmaeidae) and *Amblyseius gossipi* Elbadry (Phytoseiidae)	Prey mite was found to be an adequate food source for the development and reproduction of both predatory mite species.
			Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	*T. casei* was not suitable as food source as juveniles could not develop to adulthood and egg production was absent.
			Vila and Griffiths (2011)	*Transeius montdorensis* (Schicha) (Phytoseiidae)	Performance of the predatory mite on the prey mite was superior as compared to *Thyreophagus entomophagus*.

(*Continued*)

TABLE 7.4 (Continued)

Cohort	Family	Prey mite species	References	Predatory mites	Findings
		Acarus siro L.	Barker (1991)	*Cheyletus eruditus* (Schrank) (Cheyletidae)	A laboratory colony of *C. eruditus* was maintained on *A. siro*.
			Castagnoli et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	Juvenile development and egg production were possible, but considerably slower and lower, respectively, as compared to *L. destructor* and *G. domesticus*. Long-term rearing of *N. californicus* on this prey mite was not possible.
			Simoni et al. (2006)	*N. californicus* (McGregor) (Phytoseiidae)	The intrinsic rate of increase was significantly lower than that *of L. destructor*. Multiple generation rearing of *N. californicus* on *A. siro* showed a suboptimal predator populations.
			Cebolla et al. (2009)	*Cheyletus malaccensis* Oudemans (Cheyletidae)	Intermediate intrinsic rate of increase was obtained on *A. siro*, whereas a higher rm was observed when fed *T. putrescentiae*.
			Cakmak and da Silva (2018)	*Hypoaspis larvicolus* Joharchi and Halliday (Laelapidae)	Predatory mite showed lower population growth on *A. siro* as compared *C. lactis*, but a higher population growth as compared to *T. putrescentiae*.
		Acarus farris (Oudemans)	Ramakers and Van Lieburg (1982)	*N. cucumeris* (Oudemans), *Neoseiulus barkeri* Hughes, *Amblyseius andersoni* (Chant) (Phytoseiidae)	Mass-rearing of all three predatory mite species was possible.
		Sancassania polyphyllae Zachvatkin	Cakmak and da Silva (2018)	*Hypoaspis larvicolus* (Laelapidae)	Predatory mites did not feed on *S. polyphyllae*.
	Suidasiidae	*Suidasia nesbitti* (Hughes)	Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed high daily oviposition as compared to other tested astigmatid prey mites.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed an intermediate daily oviposition rate as compared to other astigmatid prey mites.
		Suidasia medanensis (=*pontifica*) Oudemans	Smytheman, (2011)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Predatory mite could be successfully produced on the prey mite.
			Midthassel et al. (2013)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Predatory mite could successfully complete the life cycle on the prey mite.
			Barbosa and de Moraes (2015)	*Neoseiulus barkeri* Hughes, *Iphiseiodes zuluagai* Denmark and Muma, *N. californicus* (McGregor) and *Euseius concordis* (Chant) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition as compared to other tested astigmatid prey mites.
			Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	Predatory mites showed intermediate daily oviposition rate as compared to other astigmatid prey mites.

	Winterschmittidae	*Acalvolia squamata* (Oudemans)	Massaro et al. (2016)	*Amblyseius tamatavensis* Blommers, *Euseius concordis* (Chant) and *Neoseiulus anonymus* (Chant and Baker) (Phytoseiidae)	For all three predatory mites species, *A. squamata* was an intermediate food source in terms of egg production as compared to either other prey mites or pollen.
		Oulenziella (Oulenzia) bakeri (Hughes)	Zhu et al. (2019)	*N. californicus* (McGregor) (Phytoseiidae)	Predatory mite was able to reproduce at the same rate as its natural prey, *T. urticae*, when densities of *O. bakeri* were sufficiently high.
Prostigmata	Tarsonemidae	*Tarsonemus fusarii* Cooreman	Vangansbeke et al. (2020)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Oviposition of the predatory mite on the prey mite was equal to a diet on *Carpoglyphus lactis* and higher than when fed on a diet on *Thyreophagus entomophagus*.
			Vangansbeke et al. (2021)	*N. californicus* (McGregor) (Phytoseiidae)	Oviposition of the predatory mite was equally high as compared to its natural prey, *Tetranychus urticae*.
		Tarsonemus confusus Ewing	Vangansbeke et al. (2020)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Oviposition of the predatory mite on the prey mite was equally high than a diet on *Tarsonemus fusarii*.

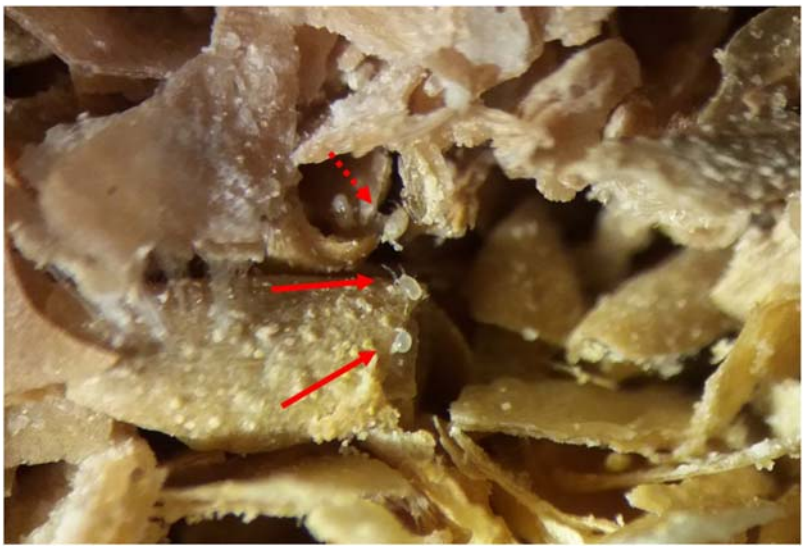

FIGURE 7.2 Close-up of a rearing medium containing wheat bran, the prey mite *Carpoglyphus lactis* (upper dotted arrow), a juvenile predatory mite *Amblyseius swirskii* (middle solid arrow) and an *Amblyseius swirskii* egg (bottom solid arrow).

relative humidity and aeration to maximize population growth (Gaede, 1992; Ramakers and Van Lieburg, 1982). Such high relative humidity stimulates fungal growth, which could affect the predators' performance and production yields (McGregor et al., 2020). The presence of the astigmatid mites has a two-fold benefit toward fungal control: direct feeding on the fungal mycelia and indirect via the production of antifungal compounds. All mites belonging to the cohort of Astigmata possess a pair of opisthonotal glands that emit a range of volatile semiochemicals with a different set of functions (Kuwahara, 2004). Those volatile compounds may act as alarm, aggregation or sex pheromone. The behavioral response depends on the mite species and concentration (Kuwahara et al., 1980a; Kuwahara, 2004; Midthassel et al., 2016). One such volatile is citral, which is a mix of its two isomers geranial (*trans*-citral) and neral (*cis*-citral) was shown to have antifungal and antimicrobial properties (Matsumoto et al., 1979; Onawunmi, 1989; OuYang et al., 2018).

The volatiles elicited by Astigmata upon predator attack can have a repellent effect (Midthassel et al., 2016, 2014), with predatory mites showing recoiling behavior (Fidgett and Stinson, 2008). This repellent behavior was also shown in the dose-response experiments of *A. swirskii* when exposed to adults of the astigmatid *Suidasia medanensis* Oudemans (Midthassel et al., 2016). When adults were freeze-killed, no such repellent effects were observed. Killing astigmatids by, for example, freezing, can overcome the deleterious effects of the physical and chemical defense mechanisms in astigmatid prey mites (Albuquerque and de Moraes, 2008; Vila and Griffiths, 2011). Feeding with dead or immobilized prey mites also facilitates the rearing of phytoseiid predatory mite species that are sensitive to disturbance by overcrowding such as *A. limonicus* or to reach higher densities of predatory mites (predatory mites per gram or per liter rearing substrate) (Bolckmans et al., 2013; Vila and Griffiths, 2011). Recently, Tabic et al. (2019) and Tabic et al. (2021) published a method for mass-rearing selected strains of *Phytoseiulus* spp. on dead prey mites, which revolutionizes its current greenhouse-based mass rearing.

Recently, other nonastigmatid prey mites were proposed as a food source for the mass-production of predatory mites. The tarsonemid mite *Tarsonemus fusarii* Cooreman belongs to the order of Prostigmata. This mite can be produced at high densities on fungal food sources and showed to be an excellent food source for *A. swirskii* and *N. californicus* (Geeraerts, 1974; Vangansbeke et al., 2021, 2020). Other tarsonemids, such as *Tarsonemus confusus* Ewing were also found to be a suitable prey mite for *N. barkeri* (Li et al., 2018) and *A. swirskii* (Vangansbeke et al., 2020). A major advantage of using tarsonemids is that all prey stages are easy to subdue (Li et al., 2018) and there are, in contrast to astigmatid prey mites, no reports about the presence of chemical defenses (Vangansbeke et al., 2020).

Based on the current state of affairs, it can be safely hypothesized that it is possible to find a suitable prey mite species or to improve its suitability by killing or immobilizing or by optimizing its nutritional value through modifying its food composition for economically mass rearing each predatory mite species.

7.6.2 Other factitious food

Factitious foods can be defined as food sources that are normally not encountered/attacked by the predator in their natural habitat (De Clercq, 2004). Some of them can be used as factitious rearing food. An overview of different factitious foods that were found to be suitable for sustaining reproduction in phytoseiids is shown in Table 7.5.

TABLE 7.5 Exemplary studies testing factitious foods for predatory mites.

Factitious prey				References	Predatory mites	Findings
Order	Family	Species	Prey stage			
Diptera	Tephritidae	*Bactrocera zonata* (Saunders)	Eggs	Momen et al. (2016)	*Amblyseius largoensis* (Muma), *Neoseiulus barkeri* (Hughes), *Amblyseius swirskii* (Athias-Henriot), *Proprioseiopsis kadii* (El-Halawany and Abdel-Samad) and *Cydnosus negevi* (Swirski and Amatai) (Phytoseiidae)	Alive *Bactrocera zonata* eggs were a suitable factitious prey for *A. largoensis*, *N. barkeri* and *P. kadii*, but *A. swirskii* and *C. negevi* failed to develop on this food source.
		Ceratitis capitata Wiedemann	Eggs	Vangansbeke et al. (2014a)	*Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Life table parameters of *A. limonicus* on frozen *C. capitata* eggs were low as compared to other factitious and natural food sources.
Lepidoptera	Tortricidae	*Cydia pomonella (L.)*	Eggs	Goleva et al. (2015)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Immature *A. swirskii* survived well and could develop on alive *C. pomonella* eggs.
	Gelechiidae	*Phthorimaea operculella* (Zeller)	Eggs	El-Sawi and Momen (2005)	*A. swirskii* (Athias-Henriot), *N. californicus* McGregor, *Typhlodromus balanites* El-Badry, *Paraseiulus talbii* (Athias-Henriot) and *Typhlodromus transvaalensis* (Nesbitt) (Phytoseiidae)	Alive eggs of *P. operculella* supported the development and reproduction of *A. swirskii* and *N. californicus*, but not of the other phytoseiids tested.
	Noctuidae	*Spodoptera littoralis*	Eggs	El-Sawi and Momen (2005)	*A. swirskii* (Athias-Henriot), *N. californicus* McGregor, *Typhlodromus balanites* El-Badry, *Paraseiulus talbii* (Athias-Henriot) and *Typhlodromus transvaalensis* (Nesbitt) (Phytoseiidae)	Alive eggs of *P. operculella* supported the development and reproduction of *A. swirskii* and *N. californicus*, but not of the other phytoseiids tested.
	Pyralidae	*Ephestia kuehniella* Zeller	Eggs	Vantornhout et al. (2004)	*Iphiseius degenerans* (Berlese) (Phytoseiidae)	Frozen eggs of *E. kuehniella* were found to be a good food source for *I. degenerans*.
				Momen and El-Laithy (2007)	*Neoseiulus barkeri* (Hughes), *Typhlodromus balanites* El-Badry *and Amblyseius zaheri* Yousef & El-Borolossy (Phytoseiidae)	Frozen eggs of *E. kuehniella* supported the development and reproduction of *N. barkeri* and *A. zaheri*, but *T. balanites* failed to develop on this food source.
				Vangansbeke et al. (2014a)	*Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Frozen eggs of *E. kuehniella* offered on a plant leaf disc, allowed juvenile survival, development and reproduction for *A. limonicus*, whereas juvenile survival on artificial Munger cells was low.
				Nguyen et al. (2014a)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	Frozen eggs of *E. kuehniella* were found to be a suitable food source for multiple generations for *A. swirskii*.
				Liu and Zhang (2017)	*Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Most immature *A. limonicus* were not able to pierce the frozen eggs of *E. kuehniella*.

(*Continued*)

TABLE 7.5 (Continued)

Factitious prey				References	Predatory mites	Findings
Order	Family	Species	Prey stage			
				Leman and Messelink (2015)	*A. swirskii* (Athias-Henriot) and *Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Sterilized eggs of *E. kuehniella* resulted in a high oviposition rate for *A. swirskii*, but not for *A. limonicus*.
				Delisle et al. (2015)	*A. swirskii* (Athias-Henriot) and *N. cucumeris* Oudemans(Phytoseiidae)	Frozen eggs of *E. kuehniella* were found to be a good food source for both *A. swirskii* and *N. cucumeris*.
				Nemati et al. (2019)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	UV-irradiated *E. kuehniella* eggs were found to be a good food source for *A. swirskii*.
				Navarro-Campos et al. (2016)	*Gaeolaelaps aculeifer* Canestrini and *Stratiolaelaps scimitus* (Womersley) (Laelapidae)	Reproduction of *G. aculeifer* and *S. scimitus* was equally high on frozen *E. kuehniella* eggs than on its natural thrips prey.
				Momen et al. (2020)	*Neoseiulus barkeri* Hughes (Phytoseiidae)	Frozen eggs of *E. kuehniella* were a superior food source as compared to the factitious prey mite *M. fungivorus*
Anostraca	Artemiidae	*Artemia franciscana* Kellogg	Cysts	Riahi et al. (2018)	*Typhlodromus bagdasarjani* Wainstein and Arutunjan (Phytoseiidae)	Decapsulated cysts of *A. franciscana* were found to be a good food source for *T. bagdasarjani*.
				Vantornhout et al. (2004)	*I. degenerans* (Berlese) (Phytoseiidae)	Encapsulated cysts of *A. franciscana* did not allow development, whereas decapsulated cysts were found to be a good food source for *I. degenerans*.
				Nguyen et al. (2013)	*Amblyseius swirskii* (Athias-Henriot) (Phytoseiidae)	Decapsulated cysts of *A. franciscana* were found to be a good food source for *A. swirskii* for multiple generations.
				Vangansbeke et al. (2014a)	*Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Decapsulated cysts of *A. franciscana* were found to be a good food source for *A. limonicus*.
				Leman and Messelink (2015)	*A. swirskii* (Athias-Henriot) and *Amblydromalus limonicus* (Garman and McGregor) (Phytoseiidae)	Oviposition of both *A. swirskii* and *A. limonicus* on decapsulated cysts was found to be lower than control diets on pollen.
				Vangansbeke et al. (2016a)	*A. swirskii* (Athias-Henriot) (Phytoseiidae)	High quality decapsulated cysts of *A. franciscana* were found to be a good food source for *A. swirskii*, whereas the performance on lower quality cysts was inferior.
				Navarro-Campos et al. (2016)	*Gaeolaelaps aculeifer* Canestrini and *Stratiolaelaps scimitus* (Womersley) (Laelapidae)	Reproduction of *G. aculeifer* and *S. scimitus* was equally high on hydrated encapsulated *A. franciscana* cysts as on its natural thrips prey.

Eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) have been tested as factitious food for several phytoseiids. In the case of *A. limonicus*, juveniles were found to be unable to pierce the flour moth eggs in the study of Liu and Zhang (2017), most likely caused by hardening of the egg chorion under the tested low relative air humidity conditions (ca. 50% RH). At higher relative air humidity (ca. 90% RH), the survival rates of immature *A. limonicus* fed on *E. kuehniella* eggs increased (Liu and Zhang, 2017). Juvenile survival of *A. limonicus*, fed *E. kuehniella* eggs and with access to free water, was lower when reared on arenas with artificial substrates than on a bean leaf arena (Vangansbeke et al., 2014b). Similarly, the oviposition rate of *A. limonicus* feeding on *E. kuehniella* eggs was low as compared to mites feeding on a *Typha* pollen diet, whereas an equally high oviposition was obtained for *A. swirskii* when fed *E. kuehniella* eggs or *Typha* pollen (Leman and Messelink, 2015).

Cysts of the brine shrimp *Artemia franciscana* Kellogg are harvested from salt lakes worldwide for use as food in aquaculture. The cysts are produced when the environmental conditions are no longer optimal to produce free-swimming nauplii (ovovivipary). In that case, brine shrimps produce dormant cysts with a thick shell (ovovivipary) (Van Stappen, 1996). *Artemia* cysts were found to be an excellent food source for phytoseiid predatory mites (Table 7.5). However, there are a few essential conditions. First, cysts need to be decapsulated, which can be done by following a simple procedure (Sorgeloos et al., 1977), to allow the predatory mites to feed on them. In the study of Vantornhout et al. (2004), *I. degenerans* was not able to develop on encapsulated *Artemia* cysts, whereas decapsulated ones allowed complete development. Secondly, cysts need to be hydrated to allow the predatory mites to ingest the cysts' content (Grosman et al., 2019). Hydration can be done actively by immersing the cysts in water (Leman and Messelink, 2015), or passively by exposing the cysts to high relative humidity conditions ($>90\%$ RH) (Vangansbeke et al., 2016a). Finally, *Artemia* cysts can vary substantially in their nutritional quality, depending on the strain and origin (De Clercq et al., 2005; Grosman et al., 2019). Commercially available decapsulated cysts, marketed to support the establishment of mainly *Macrolophus* (Hemiptera: Miridae) species (Brenard et al., 2019), yielded a ca. 30% lower intrinsic rate of increase for *A. swirskii* as compared to high-quality decapsulated cysts (Vangansbeke et al., 2016b).

Some factitious foods proved to be excellent food sources, with comparable life table parameters as compared to natural prey. Hence, many studies proposed moth eggs or *Artemia* cysts for use in mass-production systems. One major constraint of using the above-shown factitious foods is the high production/purchase price as compared to producing astigmatid prey mites. In contrast, factitious prey mites can be produced at a substantially lower cost price and therefore remain the preferred food source for commercial large-scale production of predatory mites.

Finally, as mentioned earlier, the production of phytoseiid predatory mites requires high relative humidity conditions. When providing factitious foods under such climatic conditions, the nutritional quality decreases rapidly and fungal growth can impede a continuous mass-production system. This latter issue is not the case in mass-rearing conditions containing factitious prey mites since the prey mites keep the fungal growth at bay.

7.7 System 5: predatory mites grown on plants or parts thereof using pollen

This type of mite production system is especially suitable for mites that cannot be reared, or at least not efficiently, on factitious prey mites or spider mites, thus mostly Type IV predatory mites (McMurtry et al., 2013). Besides their excellent performance on plant pollen, many of the predators within this group cannot be reared off-plant as they require plant material for a water source (Adar et al., 2012). Therefore, this system is suitable for *Euseius* and *Iphiseius* species.

Mass rearing on whole plants with pollen was proposed by Ramakers and Voet (1995). For this purpose, castor bean plant plants, *Ricinus communis* L. (Malpighiales: Euphorbiaceae) were proposed as the plants grow fast and exhibit both extrafloral nectaries and pollen. The male flowers produce an abundance of pollen, which drop to the lower leaves serving as food for the predators. The thrips predator *I. degenerans* (Berlese) was shown to benefit from both pollen and the extrafloral nectar, with higher oviposition rates when offered *R. communis* pollen with nectar as the pollen-diet alone (van Rijn and Tanigoshi, 1999). When *R. communis* plants are infested with *I. degenerans*, the extrafloral nectaries allow the predators to survive. Upon emergence of the flowers, the predatory mite numbers increase exponentially. In the study of Ramakers and Voet (1995) 11,500 mobile stages of *I. degenerans* were counted on a four-month-old castor bean plant (infestation number not known).

Jack bean, *Canavalia ensiformes* L. (Fabales: Fabaceae) was tested as a plant source for *Euseius concordis* (Chant) (de Figueiredo et al., 2018). In this case, cohorts of 15 young bean plants (ca. 15 cm in height in 1 m^2) were sprinkled with about 3 mg of *Typha domingensis* Persoon (Poales: Typhaceae) pollen every third day. Three weeks later the initial population of 300 adult *E. concordis* reached nearly 6000 mites.

A further optimization step toward using plant material would include detaching the leaves, as it would eliminate potting soil or other growing substrates from the production of predatory mites. However, the loss of leaf turgor requires frequent replacement of the leaves (de Figueiredo et al., 2018), which is not feasible. Therefore, it was proposed to use leaves of succulent plants (Wäckers and Arijs, 2014). The use of the succulent plant *Crassula* sp. (Saxifragales: Crassulaceae) and about 0.2 mg of *Typha* sp. pollen allowed an initial population of *Euseius stipulatus* (Athias-Henriot) of 200 mobiles to increase to about 1100 mites in two weeks. Alternatively, detached leaves can be placed on moist cotton or agar. Kostiainen and Hoy (1994) developed a method to produce *Euseius finlandicus* (Oudemans) on bean leaves placed on moist cotton using cattail pollen as a food source. Although such rearing methods using leaf arenas might be practical for laboratory colonies, they are not feasible for large scale industrial production.

7.8 System 6: predatory mites are grown on artificial diet

It always seems impossible, until it is done.

— Nelson Mandela

The complexity of mass-rearing systems can be reduced, and their controllability increased significantly by eliminating trophic levels, such as plants growing prey food on and/or the prey food itself (Morales-Ramos et al., 2014). Artificial diets have been suggested as the ultimate "silver-bullet" solution to optimize the cost-effectiveness of mass production of beneficial arthropods (Cohen, 2015; Riddick, 2009). Whereas many artificial diets have been developed for insect predators (Cohen and Smith, 1998; Riddick, 2009), relatively few successful artificial diets are reported for predatory mites (Kennett and Hamai, 1980; Nguyen et al., 2013).

One of the first reports of an artificial diet formulated for a phytoseiid was a study by McMurtry and Scriven (1962), whose initial set-up was to develop a method to improve the quality of *Euseius hibisci* (Chant) and *Amblydromalus limonicus* Garman and McGregor during transport. Therefore, the researchers initially used a layer of agar (2% agar−98% water) combined with tetranychid prey mites. In a further step, yeast hydrolysate and honey addition "markedly increased reproduction of *E. hibisci*". Triggered by this observation the same authors subsequently tested different combinations of sucrose, molasses and yeast hydrolysate for *A. limonicus*, *E. hibisci*, *Galendromus occidentalis* (Nesbitt), and *Typhlodromus rickeri* Chant (McMurtry and Scriven, 1966). The first two species responded well to the combinations of sugars and yeast hydrolysate, whereas none of the offered diets was suitable to allow reproduction of *G. occidentalis* and *T. rickeri*. For the spider mite specialist *P. persimilis*, Shehata and Weismann (1972) tested three different artificial diets that allowed development to adulthood but failed to produce viable eggs. Kennett and Hamai (1980) tested an artificial diet consisting of honey, yeast flakes, yeast hydrolysate, enzymatic casein hydrolysate and egg yolk for several phytoseiids with a varying degree of diet specialization. Although some phytoseiids, such as *Amblyseius largoensis* Muma and *A. limonicus*, survived and reproduced, none of the tested species could reach the reproductive output that was generated when fed their natural (spider mite or pollen) food source. Other studies testing artificial diets for phytoseiids also reported a lower survival, longer development and/or a lower reproduction as compared to natural food sources (Abou-Awad et al., 1992; Itagaki and Koyama, 1986; Ochieng' et al., 1987; Shih et al., 1993). Ogawa and Osakabe (2008) tested different diet combinations for *N. californicus* with 100% survival of juveniles when fed artificial diets consisting of honey, sucrose, tryptone, yeast extract and egg yolk. The artificial diets could keep the females alive for up to 60 days, but reproduction was practically absent. However, when switching back to a spider mite diet, reproduction resumed.

By supplemented one of the artificial diets of Ogawa and Osakabe (2008) with 20% hemolymph from the oak silkworm *Antheraea pernyi* (Guérin-Méneville), Nguyen et al. (2013) were able to obtain an intrinsic rate of increase equally high than a *C. lactis* diet for *A. swirskii*. This discovery stimulated more researchers to test different animal-derived compound in more simple artificial diets. Of the tested compounds, *E. kuehniella* eggs, *Artemia franciscana* cysts and hemolymph of black soldier fly larvae, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) (Nguyen et al., 2014a, 2015; Riahi et al., 2017) showed to have great potential boost the reproductive performance of the predatory mites (Fig. 7.3).

As was also stressed by Grenier and de Clercq (2003), multiple generations need to be tested on a specific diet in order to fully validate its potential. This issue was demonstrated by Nguyen et al. (2014a) who showed that a basic diet, which was not supplemented by either *E. kuehniella* or *A. franciscana*, could not reach the third-generation for *A. swirskii*. When *E. kuehniella* and *A. franciscana* were supplemented, a third-generation on the artificial diet could be obtained, albeit with lower r_m-values as compared to the first generation.

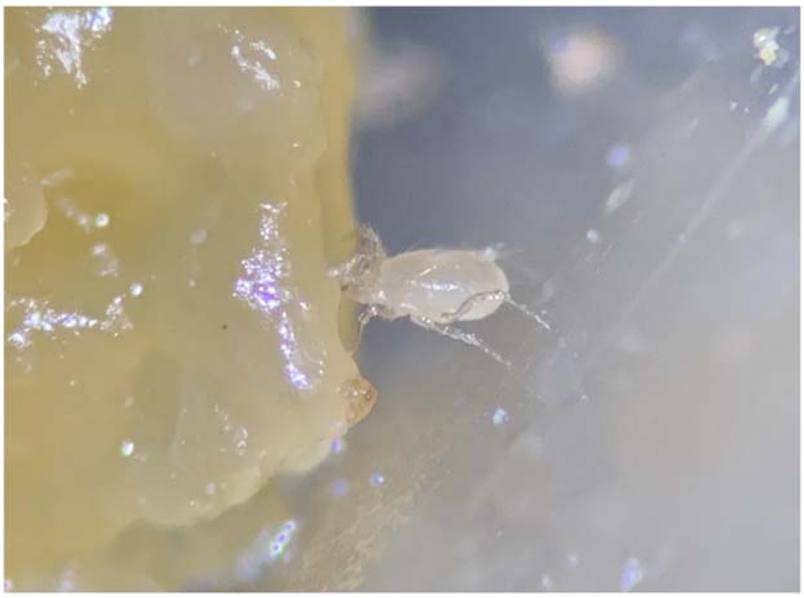

FIGURE 7.3 Amblyseius swirskii feeding on a semisolid artificial diet. *Picture credit: Dr. Duc Tung Nguyen.*

In the development of an artificial diet, it is of paramount importance to understand the feeding mechanism of the species under investigation. Like the great Allen Cohen mentioned in his highly influential book "Insect Diets: Science and Technology," it is important to understand the "feeding biology" (Cohen, 2015). Phytoseiid mites use their chelicerae to cut and pierce the cuticle of their prey after which the corniculi are partially inserted (Flechtmann and McMurtry, 1992). The above mentioned artificial diets were all offered in a semiliquid formulation to the phytoseiids. Although solid artificial diets are usually developed for insects with chewing mouthparts (Morales-Ramos et al., 2014), such diets can be used by insects with extra-oral digestion (Cohen, 2015). As suggested by Flechtmann and McMurtry (1992), phytoseiids most likely inject saliva with proteolytic enzymes into the prey. As solid, powdered or freeze-dried artificial diets have some advantages in terms of storage, application and shelf-life, Nguyen et al. (2014b) compared some freeze-dried artificial diets with their liquid counterparts for *A. swirskii*. Their results showed that freeze-drying did not affect the nutritional quality of the diets. It should be noted that the freeze-dried artificial diets were highly hygroscopic and became semisolid when offered to the predatory mite in a humid environment. The same freeze-dried artificial diet also showed potential to support *A. swirskii* population on crops in a follow-up study (Vangansbeke et al., 2016b).

More work still needs to be done to develop artificial diets for phytoseiid predatory mites for use in mass-rearing conditions. When introducing the high humidity systems, artificial diets are prone to microbial contamination. The addition of antibiotics or antifungal compounds could partly prevent this, but more research is necessary to rule out the potential negative long-term effects of such compounds.

7.8.1 From laboratory colony to mass production scale: a huge step

"It remains a fine line and a great challenge to insect diet and rearing professionals to perform the balancing act of keeping the insect well nourished and reproductively capable."

— Allen Cohen

Growing predatory mites in the laboratory is relatively easy. It requires a simple set-up and usually an easily available food source (e.g., cattail pollen) is sufficient to maintain a laboratory colony. Commercial-level mass-production of predatory mites, on the other hand, is a totally different enterprise. Upscaling of System 4 rearing systems to higher volumes for example goes hand-in-hand with challenges with humidity levels, metabolic heat, aeration and keeping fungal growth at bay (McGregor et al., 2020; Ramakers and Van Lieburg, 1982). There is an optimum in the interplay between those above factors to achieve a continuous and reliable mass-production system.

Some key factors that are challenging in large scale predatory mite production are addressed below. We will focus on the factitious prey mite rearing system (rearing System 4) as this method is most widely applied by biocontrol producers.

7.9 Prey mite

7.9.1 Suitable species

The selection of a suitable food source, here a factitious prey mite, is a first and crucial step in developing a mass-rearing system. A few important prerequisites determine the potential of a candidate prey mite.

First, the prey mite needs to be of superior nutritional quality for the reproduction and development of the phytoseiid predatory mite to allow long-term rearing. A standard screening method to evaluate the nutritional value of a food source for phytoseiids is by constructing life table parameters, which is considered an appropriate tool to predict demographic parameters (Birch, 1948; Castagnoli et al., 2006). From those life tables, valuable information can be derived such as intrinsic rate of increase (r_m), finite rate of increase (λ), net reproductive rate (R_0) mean generation time (T) and doubling time (Dt) (Maia et al., 2000). Based on this information, biocontrol producers can predict the growth of the predatory mite population. Calculating the full life tables is a time-consuming and labor-intensive activity and can last up to 2 months for phytoseiids. Therefore, Janssen and Sabelis (1992) proposed a shortcut by determining the peak oviposition rate of phytoseiids. The rate of oviposition of phytoseiids shows a steep increase to a peak, after which the oviposition rate gradually decreases (Broufas and Koveos, 2000; Nguyen et al., 2013; Park et al., 2011). This peak oviposition rate correlates with the intrinsic rate of increase (r_m) (Janssen and Sabelis, 1992) and is an easier and faster parameter to determine. Studies conducted by (Barbosa and de Moraes, 2015; Massaro et al., 2016; van Rijn and Tanigoshi, 1999) are examples of large scale screenings of food sources for phytoseiids using peak oviposition rate as a first evaluation.

Next, the selected prey mite food needs to be evaluated over multiple generations (Cohen, 2015), as some nutritional imbalances may only be revealed after multiple generations (De Clercq et al., 2005). Some food sources that were concluded to be suitable for phytoseiids in the first generation, were no longer optimal after multiple generations (Nemati et al., 2019; Nguyen et al., 2014a).

Ideally, it should be possible to mass-rear the selected prey mite species at high density on a simple, inexpensive diet, such as wheat bran, germ and flour, yeast and sugars (Fidgett and Stinson, 2008; Huang et al., 2013; Ramakers and Van Lieburg, 1982). Production of prey mites at high density combined with high population growth, reduces the required space and labor, thereby allowing a cost-effective breeding system.

Another important selection criterium for suitable prey mites is the inability (or lack thereof) to cause leaf damage. The commercial end-product of phytoseiid predatory mites usually contains a certain amount of living prey mites. This predatory-prey mite mixture is applied directly to the crop or introduced with slow-release sachets. Inevitably, some prey mites end up on the crop foliage. *Tyrophagus putrescentiae* (Schrank), which is used to mass-produce, for example, *N. cucumeris* and *Stratiolaelaps scimitus* (Womersley), is occasionally associated with plant damage depending on the prey mite strain, crop species and the climatic conditions in the crop (Czaikowska et al., 1988; Hoy, 2009; Oliveira et al., 2007; Pirayeshfar et al., 2020). A simple method for screening plant damage potential is by exposing young cucumber plants (second leaf stage) to an overdose of prey mites. One week after the inoculation, damage patterns, in the form of yellow chlorotic and necrotic spots and holes in the leaves, can be seen in prey mites with crop-damaging potential.

7.9.2 Suitable life stages

Besides offering a suitable prey mite species, the age distribution of the prey mite population has a profound effect on the predatory mite. Midthassel et al. (2013) conducted capture success trials by offering *A. swirskii* different developmental stages of the astigmatid *S. medanensis*. Eggs showed to be the easiest to kill, followed by nymphal stages. The largest developmental stage, that is, adult females, were the most difficult to subdue. When producing prey mites in a continuous system, the aim should be to reach populations with large proportions of eggs and young stages, which are the preferred prey stage for phytoseiids (Guichou et al., 2015; Li et al., 2021). Predator-prey dynamics could be different when the prey mite food is dominated by adults as compared to young stages (Midthassel et al., 2013).

7.9.3 Suitable diet/rearing substrate

The nutrient composition of prey is affected by the diet on which it feeds, which in turn has an effect on the predator (Boersma et al., 2008; Mayntz and Toft, 2001). Adapting the food of the prey mite will thus cascade bottom-up and affect the performance of the predatory mites. Changing the diet of *T. putrescentiae* had a significant impact on the performance of *N. barkeri* (Huang et al., 2013). When wheat bran, which is the basic component of astigmatid rearing media

(Ramakers and Van Lieburg, 1982), was supplemented with yeast powder or sugar in the prey mite diet, development shortened and reproduction increased significantly for the predator *N. barkeri* (Huang et al., 2013). Wheat germ and yeast addition to a wheat bran diet of *T. putrescentiae* also improved the reproductive performance and even the size of the eggs produced by *N. barkeri* (Lv et al., 2016). Sarwar et al. (2010) fed *Neoseiulus pseudolongispinosus* (Xin, Liang and Ke) with *T. putrescentiae* fed on flour of maize, soybean or wheat. The overall performance of the predator was better when its prey food was reared on wheat flour. The addition of dextrose in the diet of the astigmatids *Thyreophagus entomophagus* (Laboulbene) and *C. lactis* significantly improved the productivity of *A. swirskii* populations (Fidgett and Stinson, 2008). The oviposition rate of *A. swirskii* was higher on mixed stages of *T. putrescentiae* that were previously grown on wheat bran as compared to dog food (Pirayeshfar et al., 2020. When the fungivorous *T. fusarii* was grown on bran inoculated with the fungus *Aspergillus oryzae*, reproduction of *N. californicus* was significantly lower as compared to a diet of tarsonemids grown on *Fusarium venenatum* Nirenberg. When the tarsonemids were separated from the growing medium by sieving, the negative effect on reproduction was eliminated. This suggests that the growing media itself was causing the observed reduced reproduction in *N. californicus* (Vangansbeke et al., 2021).

7.9.4 Predator:prey ratio

Carefully balancing an optimal ratio between predatory mites and prey mites assures a maximum attainable rate of increase in the predatory mite population. When the ratio is too low, the rate of increase of the predatory mite population will decline due to starvation. When the ratio is too high, the rate of increase of the predatory mite population will decline due to overcrowding. Especially *A. limonicus* is sensitive to such density-dependent interspecific interaction. A potential solution is by offering dead (Vila and Griffiths, 2011) or immobilized prey mites (Bolckmans et al., 2013; Vila and Griffiths, 2011).

7.10 Climate management

7.10.1 Carbon dioxide concentration

Careful conditioning and sufficiently refreshing the air in the climate rooms are critical to maintain optimal conditions of air quality [(CO_2) and volatiles], temperature and relative humidity in the rearing substrate. The necessity of aeration in larger scale rearing systems was emphasized by Ramakers and Van Lieburg (1982). At high density, metabolic CO_2 produced by the prey mites can accumulate fast and cause asphyxiation of the predators within a matter of hours (Ramakers and Van Lieburg, 1982). Besides CO_2 removal, aeration is advised to reduce the amount of volatiles produced by the astigmatid prey mites present in the medium. As discussed above, astigmatid mites emit volatiles upon attack by for example phytoseiid predatory mites in a mass-production unit. For *Carpoglyphus lactis*, Kuwahara et al. (1980b) showed that the amount of citral being emitted increased with a factor of 50 when comparing post-disturbance as compared to pre-disturbance. For *Suidasia medanensis* Midthassel et al. (2016) found an increase of factor 560 after disturbance in the headspace of the rearing unit. The differential emission of citral (and other volatiles) may partly explain why some astigmatid prey mites might be found suitable when being tested in the laboratory at the individual level or in small-sized rearing containers but fail in the much larger rearing units (Pirayeshfar et al., 2020).

7.10.2 Temperature and metabolic heat

As mites are ectothermic organisms, the experienced body temperature is affected by the ambient temperature. Physiological processes underlying development, reproduction and growth are thus affected by the environmental temperature. Similar to other ectothermic arthropods (Logan et al., 2006; Shi and Ge, 2010; Wagner et al., 1984), the relationship between temperature and life history parameters of phytoseiid predatory mites follows a nonlinear curve (e.g., Lee and Gillespie, 2011; Tanigoshi et al., 1975; Tsoukanas et al., 2006; Vangansbeke et al., 2015) (Fig. 7.4).

To maximize the population growth, intuitively, one would aim to set the optimal rearing temperature ($=T_{opt}$) to T_{max} (Fig. 7.4). This would indeed be the case if the ambient temperature in the rearing medium can be maintained at exactly T_{max}. Due to metabolic heat produced by micro-organisms in the rearing medium (fermentation-like processes) and the mites themselves, the temperature can build up significantly inside a rearing container. As can be seen in Fig. 7.4, the temperature-performance rate curve declines sharply when temperatures exceed T_{max}, which results in a significant decrease in performance. Even a limited increase above T_{max}, can have drastic consequences on the performance. A small temperature variation lower than T_{max}, on the other hand, has relatively less impact. In a natural

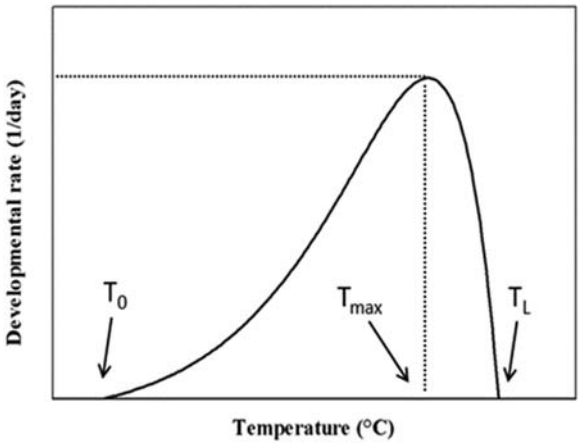

FIGURE 7.4 Example of the temperature-dependent growth curve.

environment, temperature variations are the rule rather than the exception. It is therefore believed that the overall fitness of ectotherms is higher at a temperature lower than T_{max} ("suboptimal is optimal", (Martin and Huey, 2008)). Having control over the temperature inside the rearing medium (i.e., less varation) will allow mass-producing mites at temperatures closer to T_{max}.

7.10.3 Relative humidity and substrate moisture content

Life on a plant leaf [. . .] means never having to go thirsty.

—J.A. Yoder

As discussed before, the ambient relative humidity surrounding both prey mites and predatory mites is crucial for their development as well as for the development of fungi which serve as a food source for fungivorous prey mites. It is determined by both the moisture content of the rearing substrate as well as the ambient relative humidity in the rearing room. Relative air humidity plays a pivotal role in maintaining the water balance of phytoseiid predatory mites. Phytoseiidae are small leaf-dwelling predators thriving in the thin and (usually) humid boundary layer on the underside of plant leaves. Due to their small size, mites have a large surface-area-to-volume ratio. Therefore, mites are highly vulnerable to evaporative water loss (Gefen et al., 2006; Le Hesran et al., 2019). The effect of relative humidity was demonstrated in the key study of Gaede (1992). In that study, *P. persimilis* was subjected to a range of different relative humidities to determine the so-called "critical equilibrium humidity", which is usually around 90% RH. The authors showed that below this threshold *P. persimilis* was losing water, determined by weight loss, whereas above the threshold, body weight increased could be absorbed passively from the moist ambient air. Although water can be ingested (i.e., drinking), the passive water uptake is a crucial element in maximizing the performance of the predator. It is believed that this passive water absorption is salt-driven and mouthparts are involved. Blocking the mouthparts of *G. occidentalis* with wax impeded the water uptake at high relative humidity, whereas without waxed mouthparts water uptake was not impeded (Yoder, 1998). Maintaining the relative humidity higher than the critical equilibrium humidity is one of the key factors of a successful mass-production system. For Astigmata, similar passive absorption of water is reported, albeit at a lower critical equilibrium humidity than phytoseiids (Arlian, 1989).

As there is a significant interplay between temperature and relative humidity, vapor pressure deficit (VPD in kPa) is a more preferred parameter to assess the "drying effect" of the environment (Anderson, 1936; Ferro and Chapman, 1979). For example, the drying effect on a mite at 76% RH will be higher at 30°C (~1.04 kPa), as compared to 25°C (~0.77 kPa) (Shipp and Van Houten, 1997). When assessing the evaporative water loss in phytoseiids, vapor pressure deficit is a more relevant parameter (Shipp and Van Houten, 1997; van Houten et al., 1995).

7.11 Intraspecific competition

Cannibalism is a ubiquitous phenomenon in the animal kingdom (Elgar and Crespi, 1992) and phytoseiid predatory mites are no exception [for details, see the review of Schausberger (2003)]. In high-density mass-rearing conditions, cannibalism can be a serious limitation affecting the cost-effectiveness of the production system. For example, cannibalism is one of the key reasons why mass-production of *A. limonicus* is more challenging than for example *N. cucumeris*

or *A. swirskii* (Messelink et al., 2006; Vangansbeke et al., 2014a). One way to reduce cannibalistic encounters is to provide more habitat structures where mites can shelter (Pozzebon et al., 2015). For *A. limonicus*, Lee and Zhang (2016) tested several types of rearing media in combination with *Typha* pollen as a food source. Their results clearly showed that habitat structure affects the population growth in small scale laboratory experiment, with rice husks as the preferred medium as compared to the standard used wheat bran. Likewise, millet husks also provided a preferred habitat for *A. limonicus* as compared to wheat bran in the study by Bolckmans et al. (2013). Cannibalism in *A. limonicus* was also found to be associated with diet, with females killing their newly deposited eggs when fed with some facitious foods (Vangansbeke et al., 2014a). When fed their natural preys, *F. occidentalis* and *T. vaporariorum*, no egg-cannibalism was observed. However, when fed the factitious food *E. kuehniella* and *Ceratitis capitata* Wiedemann, eggs were killed, often immediately after deposition. Strikingly, there was a lot of variation in egg-cannibalism between the tested females. When fed *E. kuehniella*, some *A. limonicus* females consumed >90% of the eggs produced, whereas other females did not engage in cannibalism (Vangansbeke et al., 2014a). In this case for *A. limonicus*, it could be interesting to select the noncannibalizing females for further testing under mass-rearing conditions. For other species of phytoseiids including *Amblyseius herbicolus* and *I. degenerans*, offering high-quality food such as *Typha* sp. pollen, can reduce the level of cannibalism (Calabuig et al., 2018; Marcossi et al., 2020). The presence of lower quality alternative food (pollen of cotton, *Gossypium hirsutum* L.), however, did not reduce cannibalism in *A. herbicolus* (Marcossi et al., 2020).

Although the immobile eggs would be the most obvious victim to cannibalize, many phytoseiid species have difficulties with piercing the egg chorion, and therefore prefer to cannibalize larvae (Schausberger, 2003). However, there can be nonconsumptive effects on the eggs after predator handling, such as delayed egg hatch. For the spider mite predator *Neoseiulus womersleyi* (Schicha), Fukuse and Yano (2019) found delayed hatching of the egg in response to mechanical stimuli (i.e., a fine brush), to simulate (conspecific) feeding attempts.

Density-dependent competition is not only limited to (attempted) cannibalism but can potentially affect juvenile survival, delayed maturity and body size (Agnew et al., 2002; Mueller, 1997). Although this is studied extensively in insects, little is known for phytoseiids, especially under high-density mass-rearing conditions.

7.12 Contamination management

Probably one of the biggest challenges for System 4 mass-rearing systems is contamination by other mite species. Especially contamination by *T. putrescentiae* in predatory mite mass-rearings on other prey mite species can trigger a rapid decline of the predatory mite population. Also, contamination with *Blattisocius* sp. can cause substantial challenges. Preventing contamination requires suitable containerization of the production, strict process quality control, highly disciplined hygiene of rearing rooms, rearing materials and personnel, and maintaining pure laboratory cultures for infestation material.

7.13 Nonphytoseiid predatory mites

So many mites, so little time!

Barry O'Connor

Besides phytoseiid predatory mites, numerous other predatory mites have been explored for their biocontrol potential [for overview, see Carrillo et al. (2015)]. However, up to date only few nonphytoseiid predatory mites have been successfully commercialized. As was pointed out by Knapp et al. (2018), the lack of knowledge on basic ecology of those mite species is a major bottleneck for their commercialization.

7.13.1 Soil predatory mites

The most important soil-dwelling predatory mite used in IPM is *S. scimitus* (*Hypoaspis miles*) (Laelapidae). This polyphagous soil predator feeds on a wide range of crop pests, including thrips (pre-) pupae (Navarro-Campos et al., 2012; Premachandra et al., 2003), bulb mites (Lesna et al., 1996), fungus gnats (Enkegaard et al., 1997; Freire et al., 2007), shore flies (Vänninen and Koskula, 2004) and nematodes (Yang et al., 2020). Mass-rearing of this soil predator primarily includes the astigmatid prey mites *A. siro* (Wright and Chambers 1994) and *T. putrescentiae* as a food source (Grosman et al., 2011; Prado Freire and De Moraes, 2007; Steiner et al., 1999) in a medium usually consisting of peat and inert material, such as rice or spelt husks or vermiculite. Those latter materials increase hiding space, while at the same time allowing mites to move around more (Prado Freire and De Moraes, 2007; Steiner et al., 1999). Sanitation

and adequate humidity levels are required to avoid asphyxiation of mites and acidification of the medium (Steiner et al., 1999). Besides *T. putrescentiae*, other tested food sources for rearing *S. scimitus* include the acarid prey mite *Sancassania* aff. *sphaerogaster* (Zachvatkin) (Cabrera et al., 2005).

Another similar soil-dwelling species is *Gaeolaelaps aculeifer* Canestrini (Laelapidae) has a similar prey spectrum, but generally higher oviposition rate as *S. scimitus* (Berndt et al., 2004). Although alternative foods like *Artemia* cysts or *Ephestia* allow a high reproduction for both *S. scimitus* and *G. aculeifer* (Navarro-Campos et al., 2016), such factitious foods are not being used in mass-rearing systems. Nematodes, on the other hand, are an interesting option to explore for mass-producing soil predatory mites as they (1) are an excellent food source for many soil-dwelling predatory mites (Azevedo et al., 2019; Heidemann et al., 2014), (2) easy and inexpensive to rear (Navarro-Campos et al., 2016) and (3) are adapted to live in moist conditions necessary to produce soil predatory mites. For this purpose, free-living saprophagous nematodes such as *Panagrellus* sp. and *Rhabditella* sp. have been successfully tested (Azevedo et al., 2019; Navarro-Campos et al., 2016). *Macrocheles robustulus* (Berlese) (Macrochelidae) is another commercially available soil-dwelling predator of thrips pupae (Messelink and van Holstein-Saj, 2008) and sciarid flies (Grosman et al., 2011) that can be produced using *C. lactis* as food mite (Knapp et al., 2018).

7.13.2 Poultry mite predators

The poultry red mite *Dermanyssus gallinae* De Geer (Dermanyssidae) is an ectoparasite causing yearly losses of about 150 million USD for the European poultry industry (Sparagano et al., 2014; van Emous, 2005). Acaricides, which used to be the prevailing control strategy, are increasingly being banned from the market. Biological solutions have been tested with a varying degree of success. The laelapid predatory mites *Androlaelaps causalis* (Laelapidae) and *G. aculeifer*, and *Cheyletus eruditus* (Cheyletidae) were able to feed on poultry red mite (Lesna et al., 2009; Maurer, 1993) and demonstrated their potential in cage trials (Lesna et al., 2012). Laboratory colonies *of A. casalis* could be maintained on the prey mites *A. siro* and *T. putrescentiae* (Dutordoir, 2013; Lesna et al., 2012).

7.13.3 Prostigmatid predators

The most studied prostigmatid predators for implementation in biological control are *Cheyletus* species. This genus is of main interest for biological control of stored food mites from the family Acaridae and Glycyphagidae (Cebolla et al., 2009; Pekár and Hubert, 2008; Žďárková, 1998). As described above, *C. eruditus* also has great potential in the control of the poultry red mite *D. gallinae* (Knapp et al., 2018; Maurer, 1993). Mass production systems for *C. eruditus* were developed by using *A. siro* by Burnett (1960) and Žďárková (1986). In the latter study, lettuce seeds as a carrier medium and a predator−prey ratio of 1:100 to 1:200 (*C. eruditus*: *A. siro*) were suggested as the recommended rearing procedure.

Recently, the interest in the iolinid predators *Homeopronematus anconai* (Baker) and *Pronematus ubiquitus* (McGregor) regained due to the persistent issues with *Aculops lycopersici* (Massee) on tomato crops (Pijnakker et al., 2020; van Houten et al., 2020; Vervaet et al., 2021). Due to their small size, *H. anconai* and *P. ubiquitus* are able to thrive on tomato plants below the sticky glandular trichome heads that hamper the movement of phytoseiids (Vervaet et al., 2021). Both iolinid predators can be easily reared in the laboratory on leaf disc arenas with *Typha* spp. pollen (Hessein and Perring, 1986; Knop and Hoy, 1983). However, developing a large-scale rearing for such predatory mites encounters the same issues as rearing a type IV phytoseiid predator, namely the necessity of leaf material (Hessein and Perring, 1988). Up to date, no commercial product of iolinid predators is available.

7.14 Diseases

As some phytoseiid rearing systems contain 3 trophic levels, disease problems can occur on all three levels: plant, feed mite (i.e., spider mite or factitious prey mite) and predatory mite. In this chapter, we will not discuss plant diseases but focus on diseases of prey mite and predatory mite. Excellent overviews on diseases in mites were written by der van Geest et al. (2000) and Bjørnson (2008) and we refer to these reviews for more details. Below, we focused on diseases more commonly encountered in mass production systems of phytoseiids and their natural and factitious prey mites. Mass-production entities contain high densities of predatory mites, which facilitate disease transmission (Goodwin, 1984).

7.14.1 Spider mites—the case of *Neozygites*

In the production of *P. persimilis* and similar "System 1" phytoseiids, high-quality spider mites are of paramount importance to produce top quality predatory mites. A frequently encountered problem in spider mite production are species of the pathogenic fungus *Neozygites* spp. (Zygomycetes: Entomophthorales). Fungi in this genus attack several small arthropods such as aphids, mealybugs and tetranychid mites such as *Tetranychus urticae* (Klingen and Westrum, 2007), *Tetranychus evansi* Baker and Pritchard (Wekesa et al., 2007), *Eutetranychus banksi* (McGregor) (Weiser and Muma, 1966), *Mononychellus tanajoa* (Bondar) (Oduor et al., 1996b), *Panonychus ulmi* (Kock) and *Eotetranychus sexmaculatus* (Riley) (Selhime and Muma, 1966).

Neozygites floridana is an obligate pathogen, which infects all stages of tetranychid mites, except eggs. Infected mites are slow-moving and can live up to 20 days. Mite cadavers look like brown, orange "mummies" filled with hyphal fungal bodies that grow inside the mite. Cadavers show "fluffy" grey mycelium growth externally. *Neozygites floridana* sporulates, that is, forms and ejects primary conidia formed on the exterior of killed mites, when a set of favorable conditions are met: (1) RH > 95% (leaf boundary layer), (2) temperature between 13°C and 29°C (optimal 25°C with no sporulation taking place above 29°C), (3) darkness for more than 6 hours (de Castro et al., 2013; Oduor et al., 1996a). Sporulation starts at 3 a.m. The primary conidia are actively ejected up to a distance of 9 mm and can be further spread by wind. It is estimated that up to 1.500−2.000 spores can be produced by a mummy/night. Most primary conidia are ejected during the first 12 hours of sporulation. Some *Neozygites* spp. can produce resting spores that can be zygospores (sexual spores) or azygospores (da Silveira Duarte et al., 2013; Westrum et al., 2014). The resting spores are mainly formed under low temperatures (<11°C) and short photoperiod (L10:D14) and can survive in the soil (dormancy).

The primary conidia germinate and produce capilliconidia, that is, infective spores. The requirements for the germination of the primary conidia are RH% above 95% and temperature between 13 and 29 °C (da Silveira Duarte et al., 2013; Wekesa et al., 2010). The germination of the primary conidia starts at approximately 4 hours after sporulation and between 70% to 90% of the primary conidia will germinate. The capilliconidia are spread by adhesion to the spider mites. The presence of the predator *P. persimilis* can increase the spread of the capilliconidia due to the increased movement of the spider mites. *P. persimilis* can also carry and thus spread the capilliconidia but is not being infected. Finally, the capilliconidia germinate on the spider mite, penetrate its body and develop hyphal bodies inside the spider mite (colonization). The percentage RH is no longer crucial in this stage.

The management of *N. floridana* is a challenging task given the speed the epizootic can occur which leaves too little margin for a response. Measures and efforts are directed to (1) avoiding the transfer of the pathogen from one batch to the other by (2) actively managing the climate in the greenhouse to avoid producing the conditions (temperature and relative humidity) that favor the sporulation of the primary conidia and (3) by using taller bean varieties to achieve better aeration of the crop and (4) by using fungicides and sulfur evaporation when necessary before the release of *P. persimilis* (Castro et al., 2016). de Castro et al. (2013) also suggested the use of artificial light to inhibit the production of primary conidia and capilliconidia although the challenge is to deliver high light intensities on the uunderside of the leaves where infected mites reside.

Tetranychid populations can also be affected by viral diseases. In 1955, Muma reported citrus red mite, *Panonychus citri* (McGregor), in Florida to die from dysentery showing black resinous material coming out of the body via the anus (Muma, 1955). Later, similar observations were made by Smith et al. (1959), with *P. citri* showing the same disease symptoms. Aqueous suspensions of tetranychid mites showing symptoms revealed spherical virus-like particles. Reed and Hall (1972) confirmed that a rod-shaped, noninclusion virus was causing the disease symptoms in *P. citri*.

7.14.2 Astigmatid prey mites

Healthy prey mite populations are a prerequisite for mass-rearing predatory mites. Without good quality prey mites, population growth and the overall fitness of the predatory mites can be compromised (Hubert et al., 2014). Relatively little information is reported on diseases in Astigmata (der van Geest et al., 2000). More studies were conducted on the astigmatids' general microbiome and their effects on the population performance (Hubert et al., 2021). Internal bacteria of *A. siro*, *L. destructor* and *T. putrescentiae* were acquired by ingestion and were detected in the digestive and reproductive tract (Hubert et al., 2012; Kopecký et al., 2014). Besides the presence of endosymbiotic bacteria, like *Cardinium* and *Wolbachia*, other bacteria detected inside the prey mites included *Bacillus*, *Staphylococcus*, *Enterobacteroaceae*, and *Bartonella*-like bacteria.

Growth curves of Astigmata in laboratory cultures show logistic growth with a more latent initial phase of low growth, followed by an exponential growth phase and finally a so-called drop phase of the culture (Eraso et al., 1997;

Klimov et al., 2019). This latter death phase is caused by the build-up of acaropathogenic bacteria present in the spent growth medium, consisting of mite cadavers, feces, and diet debris (Molva et al., 2020). For the American house dust mite, *Dermatophagoides farinae*, changes in the microbiome were associated with increasing population density of the mites. The death phase of populations of *D. farinae* was associated with the intracellular bacterium *Cardinium* being replaced with *Staphylococcus* and with the replacement of *S. cerevisiae* by several species of antagonistic fungi (*Aspergillus* sp. and *Candida* sp.) (Klimov et al., 2019). Some bacteria, such as *Kocuria rhizophila* and *Bacillus cereus*, detected in the spent growth medium were correlated with a decreased population growth of the European house dust mite *Dermatophagoides pteronyssinus* (Molva et al., 2019). When feeding on nitrogen-rich diets, *T. putrescentiae* showed "white body syndrome", which is caused by guanine accumulation in the fat body. The guanine crystals can also block the reproductive and digestive tracts. Moreover, guanine formation might be linked to a suppressed immune system (Smrz and Catska, 1989). To avoid the drop phase, such deleterious prey mite diets should be avoided. Reducing the proportion of "toxic" spent growth medium and build-up of unwanted micro-organisms can be obtained by frequently adding a substantial amount of fresh rearing medium.

Pekas et al. (2017) compared the entire microbiome of *N. cucumeris* and *T. putrescentiae* under mass-rearing conditions. The predatory mite and prey mite only shared 15% of core bacterial taxa, including *Cardinium*, *Wolbachia*, *Bartonella-like*, *Blattabacterium*-like, *Solitalea*-like, *Brevibacterium*, *Staphylococcus* sp. and *Bacillus cereus*. Interestingly, the bacterial community of *T. putrescentiae* was significantly affected by the presence of *N. cucumeris*. A higher relative abundance of *Kocuria koreensis*, *Brennaria* and *Staphylococcus saprophyticus* was found when the prey mites were obtained from mass-rearing conditions with the presence of predatory mites as compared to laboratory cultures without predators.

7.14.3 Predatory mites

Beerling and van der Geest (1991) reported a drastic decrease in the performance of *N. cucumeris* and *N. barkeri* under mass-rearing conditions. This decrease was linked to the presence of the obligatory intracellular pathogens Microsporidia, which can only survive outside the host cell as a spore. Those spores can enter a new host's body by consumption, after which the spore germinates in the gut system. The cycle restarts when new spores are being excreted, usually via the feces. Microsporidia can only be visually assessed if the infection rate is in an advanced state (Beerling and van der Geest, 1991). At this point, predators show signs of reduced mobility and whitish insides. The infections were not restricted to the predatory mites, but the prey mites *A. siro* and *T. putrescentiae* also showed similar symptoms. Although horizontal transmission from prey mites to phytoseiids predatory mites has not been demonstrated, prey mites should be maintained free from Microsporidia. Production of high-quality phytoseiids can only be ensured if the prey mite, on which it feeds, is performing optimally (Bjørnson, 2008).

Predatory mites infected with Microsporidia show reduced fitness. Olsen and Hoy (2002) found that *G. occidentalis* infected with the Microsporidium *Oligosporidium occidentalis* displayed a shorter lifespan, reduced fecundity and a sex ratio more male-biased as compared to their noninfected counterparts. When *P. persimilis* was infected with *Microsporidium phytoseiuli*, similar reduced fitness parameters were obtained, along with lower predation rates on *T. urticae* (Bjørnson and Keddie, 1999). Besides vertical transmission, horizontal pathogen transmission can occur upon direct contact with spore in the environment, direct contact with infected mites, via cannibalism of eggs and immatures or by grooming (Beerling and van der Geest, 1991; Olsen and Hoy, 2002).

Colonies of Microsporidia-infected *G. occidentalis* could be cured by rearing the mites under heat-treatment conditions of 33°C (Olsen and Hoy, 2002). When exposed to the heat treatment for one generation (egg-to-adult at 33°C), the infection rate of the offspring was lowered but not fully eliminated. However, when the predatory mite eggs were maintained under high-temperature conditions, the first generation was pathogen-free. Although this method was successful for *G. occidentalis*, it may not be suitable for other phytoseiids. For *P. persimilis*, heat treatments cause high mortality in the predatory mite and do not fully eliminate the Microsporidia (Bjørnson, 1998). An alternative method to obtain disease-free lines of predatory mites, is establishing iso-female lines. The offspring of those lines should be screened to validate if the line is disease-free. A simple way to check for Microsporidia presence is preparing smear preparations, which will show numerous spores (Beerling and van der Geest, 1991; Bjørnson, 2008). The next step is molecular screening for Microsporidia in mite productions, by isolating and sequencing the 16 S small subunit rRNA (SSU rRNA) (Baki and Bekircan, 2018; Chemurot et al., 2017; Dong et al., 2010).

In a study by Dicke et al. (2000), one out of the four population of *P. persimilis* tested did not respond to herbivore-induced plant volatiles (nonresponding (NR) line). As this lower attraction was found to be contagious, it was hypothesized this behavior was caused by a disease (Schütte et al., 1998). Most of the females in this NR line were flattened

dorsa-ventrally, showed a lower predation rate and failed to reproduce. Besides the decreased performance, females carried birefringent dumbbell-shaped crystals in their legs. Healthy females from non-NR lines also had these crystals, but only in the Malpighian tubules and the rectum (Schütte et al., 2006). Birefringent dumbbell-shaped crystals and accumulation of crystals in the digestive tract ("white gut syndrome") were also observed in *P. persimilis* (Bjørnson et al., 1997; Steiner, 1993). A larger proportion of *P. persimilis* showed white opisthosomal coloration when fed on *T. urticae* grown on bean plants fertilized with increasing concentration of 20−20−20 (N−P−K) (Bjørnson and Raworth, 2003). It is now accepted that, under normal conditions, birefrigent crystals are being excreted. However, in some cases, reduced fitness can be observed when the crystals are visible in the legs. Later, it was shown that the gram-positive, rod-shaped bacterium *Acaricomes phytoseiuli* was the causal agent of the NR syndrome (Pukall et al., 2006; Schütte et al., 2008). Up to date, no curative treatments for diseased phytoseiid rearings exist. A preventative approach, with introducing disease-free line regularly is therefore advised.

7.15 Challenges and future prospects

Don't be trapped by dogma-which is living with the results of other people's thinking.

—Steve Jobs

The cost-effectiveness of a high-density large-scale production system on inexpensive factitious prey mites is key to the success of predatory mites in IPM in protected cropping systems (Calvo et al., 2015). However, there are still a lot of possibilities to further optimize the existing production systems.

7.15.1 Off-plant rearing systems for types I and IV predatory mites

Until recently, it was believed that Type I specialist tetranychid predators could only be mass-produced on *T. urticae* that in turn was reared on plants. By selecting for feeding on astigmatid prey mites, Tabic et al. (2019) succeeded in allowing *P. persimilis* to reproduce for at least 2 generations on frozen *C. lactis* and feeding on other astigmatid species was also observed. This selected strain was also capable of reproducing on frozen *A. swirskii*. Although the growth of the predator populations (finite rate of increase $\lambda = 1.15-1.2$) did not reach the level of a standard System 1 production system ($\lambda = 1.4-1.5$, Galazzi and Nicoli (1996)), the scalability of such a factitious prey mite system for *Phytoseiulus* predators would be a major advantage in terms of reducing costs and increasing reliability.

The necessity for plant material for mass-rearing and the associated production costs are determining reasons why some predatory mite species (e.g., *Euseius* sp., iolinid predatory mites) have not yet entered the market or are no longer commercially available. Developing a so-called "artificial leaf" or another way to provide water while eliminating the need for plant material would open the doors for the commercialization of a wider range of predatory mites. Alternatively, specific strain selection and/or breeding could focus on the redundancy of leaf material.

7.15.2 Artificial diets

Artificial diets could eliminate the necessity for facitious prey mites. These latter food sources demand space and labor, which could be reduced drastically when using an artificial diet (Kennett and Hamai, 1980; Morales-Ramos et al., 2014). Furthermore, the production of astigmatid mites can occasionally cause health problems for workers in the prey mite production facilities due to mite-related allergens (Fernandez-Caldas et al., 2007). Although some promising results were reported, artificial diets are not implemented in commercial mass-production of predatory mites.

A major advantage of using alive prey mites is the possibility to develop controlled-release sachets. For many growers, such sachets are a preferred release method for introducing predatory mites into their crops (Buitenhuis et al., 2014; Sampson 1998). The food mix inside the sachets allows the factitious prey mites to reproduce and thereby continue to serve as food for the predatory mites for multiple weeks. Developing sachets from a mass-production system using only artificial diets will be challenging. For example, artificial diets tend to decrease in their nutritional quality due to microbial degradation (Cohen, 2015). Preservatives could reduce or eliminate such diet degradation. However, no information is available on the (long-term) effects of preservative addition to artificial diets for predatory mites.

The majority of artificial diets, which yielded a good reproductive outcome for phytoseiids, consisted of an animal-derived component, such as *Artemia* cysts or insect hemolymph (Nguyen et al., 2013, 2015; Riahi et al., 2017). Therefore, it is difficult to evaluate the value of the basic diet components. Hence, a classical deletion-addition approach could reveal the necessity of the different diet components (Cohen 2001).

7.15.3 Role of endosymbionts

The characterization of the microbiome of both predatory mites and prey mites along with understanding the interaction thereof could potentially acquire useful information to further increase the efficiency of the mass-rearing system (Pekas et al., 2017).

Endosymbionts can have a profound impact on the reproductive performance and other physiological traits of arthropods (Duron et al., 2008; Eleftherianos et al., 2013; Stouthamer et al., 1999), including phytoseiid predatory mites (Enigl and Schausberger, 2007; Zchori-Fein and Perlman, 2004). Some bacteria, like *Cardinium*, *Wolbachia* and *Spiroplasma* have received a great deal of attention due to their potential impact on the mite's reproductive performance. *Cardinium* was responsible for an increase in the reproduction of *G. occidentalis* (Weeks and Stouthamer, 2004) and is believed to be essential for mite nutrition (Penz et al., 2012). However, more fundamental research is required to gain knowledge on the impact of endosymbionts.

Ultimately, providing a probiotic cocktail might increase the performance of both predatory and prey mite populations. Moreover, such a probiotic treatment might also act as a buffer to minimize the effects of potentially harmful microorganisms.

7.15.4 Automation

The level of automation in arthropod rearing companies is generally low, which results in low productivity (Veldkamp and Bosch, 2015). This rearing cost per unit can be decreased significantly by (1) providing cheaper food sources, (2) by producing larger quantities (economy of scale), and (3) by increasing the level of automation (Cohen, 2015). Finding the balance between those three factors without jeopardizing the quality of the produced biocontrol agents toward field performance is one of the main challenges biocontrol producers are dealing with.

For the past two decades, insect rearing gained a great deal of attention for the production of insects for feed and food (Rumpold and Schlüter, 2013; van Huis, 2013). Biocontrol producers can gain a lot of knowledge from such insect procedures, as the level of automation in some of these companies is more advanced.

A plethora of parameters affects the production output of insects and mites, including ventilation, temperature, humidity, CO_2, light spectrum and intensity. Therefore, a great deal of data can be collected to analyze and identify the driving parameters. However, analyzing such large amounts of data can be highly time-consuming. For this purpose, artificial intelligence and machine learning would be an interesting approach to further optimize the level of automation and decision-making processes (Cortes Ortiz et al., 2016).

7.15.5 Strain selection

Mites Do Things They Shouldn't.

—Dana Wrensch

Selecting or breeding for different strains can focus on two different traits. First, producers can aim to select a strain of predatory mites that allows for cost-efficient rearing. One recent example is the development of a *Phytoseiulus* strain that can reproduce on astigmatid mites (Tabic et al., 2019). Secondly, mites can be selected for specific traits that are interesting for enhanced field performance. Interesting traits to select for include adaptation to abiotic factors, such as temperature and relative humidity, selection for improved establishment on challenging host plants (e.g., tomato, cannabis) or selection for pesticide-resistant strains. Different strains of *N. californicus* were found to be differentially adapted to lower relative air humidity condition in the study by Walzer et al. (2007). A strain of *N. barkeri* adapted to high temperatures was obtained by exposing a population to a combination of long-term high-temperature exposure (heat acclimation at 35°C) and short-term heat exposures (heat hardening at 45°C for 2 hours) (Zhang et al., 2018). When grown on tomato plants for four generations, *P. persimilis* outperformed the populations that were reared on bean plants with a faster predator population increase and increased spider mite control in a tomato crop (Drukker et al., 1997). Similarly, selective breeding of *A. limonicus* on tomato plants for multiple generations allowed a better establishment than the nonselected line (van Houten et al., 2017). As mass-producing predatory mites on tomato plants are economically challenging, a key question is how long the predators maintain their acquired tomato-adapted traits when moved to off-plant mass-rearing conditions. Phytoseiid predatory mites can acquire resistance against pesticides by (1) years of exposure to pesticides under field conditions (Duso et al., 2020) or (2) by selective breeding for resistance against specific pesticides (Strickler and Croft, 1982). Although it is an ongoing debate on whether this a sustainable

approach to consider for further research, resistant phytoseiids can be useful in IPM programs, especially in outdoor crops (Duso et al., 2020; Hoy, 1985).

7.15.6 Genetic variation

Long-term and high-density mass production of biological control agents are associated with inadvertent selection of certain traits, inbreeding and genetic drift, which can lead to loss of genetic variability, loss of fitness and reduced performance (Mackauer, 1976; Rasmussen et al., 2018). Inbreeding effects can already be observed in small-scale laboratory cultures, with increased mortality and lower viability in *P. persimilis* and *N. fallacis* (Poe and Enns, 1970). Paspati et al. (2019) investigated the effects of mass-production on *A. swirskii*. The commercial strain of *A. swirskii* was about 2.5 times less heterozygous than their wild strain counterparts, indicating reduced genetic variation. Although, up to date, there is no indication whether this reduced genetic variability may result in reduced biocontrol efficiency in the field, this is a topic that merits further investigation.

When establishing a population of biocontrol agents with a limited number of individuals, there is a risk that the genetic variability of this founder population is too low and maybe further reduced under a mass-rearing bottleneck. Therefore, it is advised to introduce new genetic variation from natural population on a regular basis (Nunney, 2003). Important to highlight, though, that introducing material from the wild also entails risks such as contamination with other species and diseases.

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Chapter 8

Artificial diet development for entomophagous arthropods

Juan A. Morales-Ramos[1], M. Guadalupe Rojas[1], Thomas A. Coudron[2], Man P. Huynh[3,4], Deyu Zou[5] and Kent S. Shelby[2]

[1]*Biological Control of Pest Research Unit, National Biological Control Laboratory, USDA-Agricultural Research Service, Stoneville, MS, United States,* [2]*Biological Control of Insects Research Laboratory, USDA-Agricultural Research Service, Columbia, MO, United States,* [3]*Division of Plant Science & Technology, University of Missouri, Columbia, MO, United States,* [4]*Department of Plant Protection, Can Tho University, Can Tho, Vietnam,* [5]*Biological Control of Insect Research Laboratory, Institute of Plant Protection, Tianjin Academy of Agricultural Sciences, Tianjin, P.R. China*

8.1 Introduction

Artificial diets were first developed as tools for entomological research and quickly evolved as aids for bioassay of potential insecticides, entomopathogens, and plant resistance characteristics (Stone and Sims, 1992; Cohen, 2001; Grenier, 2009). Their potential was soon acknowledged as tools for insect control in sterile male release and augmentative biological control strategies (King et al., 1985; Knipling, 1992; Cohen, 2001, 2004; Grenier, 2009). The most successful mass-rearing programs based on artificial diets were achieved with herbivorous insects produced for sterile male releases, entomopathogen mass production, or as host or prey for the rearing of entomophagous insects (King and Leppla, 1984). Developing artificial diets for entomophagous arthropods has been a more challenging enterprise and will be the focus of this chapter.

Artificial diets are critical to the advancement of mass production and eventual commercialization of entomophagous arthropods as biological control agents (Yazlovetsky, 1992). Rearing parasitoids and predators in vivo requires the rearing of multiple organisms as prey or host species must be simultaneously produced to feed the entomophagous colony. Mass rearing is a complex enterprise and its complexity multiplies as more than one species must be reared. Artificial diets are intended to eliminate the need to rear host (or prey) species thereby reducing the complexity of the system to manageable levels. King et al. (1985) considered the development of artificial diets a requirement for the success of augmentative biological control. Cohen (1992) considered the lack of suitable artificial diets the greatest barrier to the mass production of entomophagous insects. However, 17 years later few artificial diets have been utilized successfully and consistently in the commercial mass production of entomophagous arthropods (Grenier, 2009). Riddick (2009) concludes in his review that most existing artificial diets were inferior to natural or factitious prey in producing high-quality predators. The lack of rigorous artificial diet evaluation has been considered an important factor for the low success of artificial diets to date (Grenier and De Clercq, 2003; Riddick, 2009).

Artificial diets have played a very important role in advancing the science of entomology. Cohen (2001) estimated that artificial diets were used in 45% of articles published in journals of the Entomological Society of America (from 1998 to 2000), which required some type of insect rearing. However, their promising potential in augmentative biological control remains mostly unfulfilled. In this chapter, we will focus on the problems of commercial implementation of artificial diets for entomophagous arthropods and potential ways to solve them. We will not try to enumerate and describe the large number of artificial diets that have been produced in the last 50 years. Excellent reviews on artificial diets for entomophagous arthropods have been published (Grenier et al., 1994; Etzel and Legner, 1999; Thompson, 1999; Thompson and Hagen, 1999; Grenier, 2009; Riddick, 2009).

Artificial diets for entomophagous arthropods have not always been developed with the intent for use in commercial mass production but rather as means to facilitate biological research (Cohen, 2001). In most cases, published artificial diets for entomophagous arthropods have not been developed to the level required for commercial application and still may be seen as a work in progress. Successful diets are often difficult and expensive to produce in large quantities

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requiring the use of crude food ingredients and costly media for encapsulation. Most published artificial diets that have been effective for producing healthy arthropods are still unfinished when it comes to commercial use.

8.1.1 Levels of development

Artificial diets are classified as holidic, meridic, and oligidic according to the level of chemical definition as fully chemically defined, partially chemically defined, or totally undefined, respectively (Cohen, 2004, 2015). Meridic artificial diets are additionally defined as lacking any insect components (Grenier and De Clercq, 2003). If artificial diets are to play an important role in the mass production of natural enemies, efforts in their development should have as final objectives the successful mass production of entomophagous arthropods at a cost suitable for commercial application and a process that can be automated. For conceptualization, we have organized the process of artificial diet development into 6 levels required to achieve these goals.

Level 1: Arthropod completes development and reproduces in the artificial diet formulation. Often, at this level of development, artificial diets are typically chemically defined (i.e., holidic) and arthropods are reared in small containers or Petri dishes. However, some examples of meridic and oligidic diets exist at this level of development (Dindo et al. 2001). A limited number of individuals are produced in artificial media at this level.

Level 2: Arthropod completes development and reproduces for at least 5 successive generations in artificial diet. In order to maintain a colony for multiple continuous generations, a viable population must be maintained in vitro. A viable population represents a colony size of around 100 individuals at each life stage. Some of the rearing procedures must be simplified to facilitate this level of production. For example, artificial diets may remain holidic in their composition, but in many cases, some chemically defined components are replaced by natural components for simplification.

Level 3: Arthropods are produced in artificial diet in sufficient numbers and reproducibility to make possible a statistically valid comparison of biological parameters against arthropods reared on natural hosts. The need to produce arthropods in vitro in substantial numbers (several hundred individuals) drives the development of more sophisticated rearing procedures, and the means to obtain eggs in the absence of hosts or prey are often developed at this stage. Diets are mostly meridic in composition but may still include some chemically defined components.

Level 4: Arthropods are produced in artificial diet in sufficient numbers to make experimental field releases and statistically valid field evaluations. Field releases demand the production of thousands of individuals driving the development of more sophisticated rearing procedures. Techniques for enhancing diet mixing and dispensing, oviposition, egg handling, immature feeding, adult emergence, and packing for release must be developed. At this level meridic diets with provisions for microbial contamination and storage are common.

Level 5: Arthropods are produced in artificial diet in sufficient numbers to be released in a commercial field and achieve at least substantial control of the target pest. The release of arthropods in a commercial field with the purpose of controlling a pest will demand the production of tens of thousands to hundreds of thousands of high-quality individuals. Some level of mechanization must occur to maintain that level of production. Rearing systems and diet must be entirely reliable, or the enterprise will fail. Diet composition tends to be oligidic at this level with few chemically defined components including mostly vitamins and some amino acids. The diet quality at this level must be such as to produce natural enemies as good or closely comparable to those reared on natural host or prey. Given that the diet must be produced in mass, the formulation must be stable enough for temporary storage.

Level 6: Production levels of arthropods in artificial diet are only limited by space and production costs are suitable for commercialization. Production capabilities at this level should reach millions of individuals. Most of the rearing procedures must be either mechanized or extremely simplified in order to attain this level of production. Expensive materials and diet ingredients are substituted with less expensive alternatives. Diets at this level are typically oligidic in composition and stable. Mass preparation of diet involves mechanized procedures and sophisticated sterilization procedures such as flash sterilization and/or extrusion. Reliable packing, shipping and field release procedures must be developed.

These levels of artificial diet development focus on how easily an arthropod can be produced in artificial media and how effective they are fulfilling their intended purpose. Efforts to develop artificial diets to the level of commercialization require much more than just perfecting the diet formulation. However, this chapter will focus exclusively on the efforts to improve and refine artificial diets for entomophagous arthropods. Implementation of rearing procedures, packing, diet preparation and dispensing, and mechanization will be left for future discussions.

Developing artificial diets from levels 1 to 6 draws various disciplines from insect nutrition and insect dietetics to food processing sciences (Singh, 1977; Cohen, 2004, 2015). Cohen (2001, 2004, 2015) considered insect nutrition and insect dietetics as two extremes of a continuum of practicality. Developing artificial diets requires knowledge of biological, behavioral, chemical, physiological, biophysical, and physical processes which interact during ingestion, digestion, absorption, and assimilation of food by insects.

8.1.2 Degrees of difficulty

In developing artificial diets all aspects of arthropod biology have relevance, but the nature of some species' biology adds difficulty to the process of artificial diet development. For example, parasitoid koinobionts require the host to be alive during their development (Askew and Shaw, 1986). Hormonal interactions among parasitoid and host play an important role in parasitoid development (Vinson, 1984; Beckage, 1985; Grenier et al., 1986). In contrast, idiobionts consume their host in a fashion more like a predator (Quicke, 1997), with the difference that parasitoids complete their development consuming one single host. These differences in biology are fundamental in determining the degree of difficulty in developing artificial diets. Reviews of artificial diets show that more success has been obtained in developing artificial diets for idiobionts than for koinobionts (Quicke, 1997; Grenier and De Clercq, 2003). Egg parasitoids, most of which are considered idiobionts, have special needs regarding osmotic balance because they develop in the liquid media (Grenier et al., 1986, 1994). However, all parasitoids have a biological characteristic that reduces the degree of difficulty in developing artificial diets. Parasitoids are able to complete development in one single host, which provides all their nutritional needs; therefore, in theory, a complete biochemical analysis of the host composition should yield the basis for a functional artificial diet. This is not always true because many parasitoids are capable of inducing changes in their host chemistry by the action of venoms (Morales-Ramos et al., 1995; Quicke, 1997). Chemical analyses of parasitized or envenomized hosts could provide better information for developing a functioning diet (Rojas et al., 1996).

Predatory arthropods not only may feed on different prey species, but also on different plant-derived foods (Jervis and Kidd, 1996; Dixon, 2000). Omnivorous predators are known to feed on plant nectar, pollen, fruit, foliage, and fungi in addition to prey (Coll and Guershon, 2002; Lundgren, 2009a,b). Feeding on nonprey items can provide significant benefits to the development, survival, and fecundity of predatory arthropods (Eubanks and Styrsky 2005; Lundgren, 2009b). Even some predatory arthropods considered selective, such as *Phytoseiulus persimilis* Athias-Henriot (Zhang, 2003), have been reported to feed on extra-floral nectar and sugary solutions that provided significant improvements in immature survival and adult fecundity in the presence of prey (Rojas and Morales-Ramos, 2008). Nonprey foods like pollen and fungi provide a great variety of nutrients including sugars (in nectars), complex carbohydrates, protein, some free amino acids, lipids, sterols, vitamins, and minerals. Pollen protein contains high amounts of proline and pollen is also a good source of phytosterols (Lundgren, 2009a,b). Fungi can provide carbohydrates, protein, essential lipids, and ergosterol (Lundgren, 2009a,b). Consumption of nonprey foods increases the complexity of the predators' diet and alters the profile of amino acids and fatty acids assimilated, which can be relevant to artificial diet development (Rojas et al., 1996; Cohen, 2004, 2015). In order to achieve an optimal artificial diet for these predators it is necessary to identify their alternate food sources, determine their composition, and the proportion at which each is consumed. The complexity of the nutritional ecology of predatory arthropods is why a chemical analysis of the prey does not always provide the basis for a good artificial diet formulation.

8.2 Arthropod nutrition

Nutrition is the most basic subject relevant to the development of artificial diets. Diets must provide all essential nutrients to allow complete development and reproduction.

8.2.1 Carbohydrate

Carbohydrates are an important carbon and energy dietary source. The most common sugars involved in arthropod nutrition are monosaccharides and disaccharides (Singh, 1977), some of which are arthropod specific (Cohen, 2004, 2015). Carbohydrates are required to produce chitin, which is an amino polysaccharide found on the exoskeleton of arthropods (Chippendale, 1978).

Typically, sugars refer to the monosaccharides- glucose, fructose, and galactose, and the disaccharides- maltose, trehalose, and sucrose. The requirements for each specific sugar vary with the species. Most insects are able to absorb and metabolize fructose and glucose, but some monosaccharides such as arabinose, ribose, xylose, and galactose, while readily absorbed, are not metabolized (Chippendale, 1978). Generalist feeders are able to digest disaccharides, such as sucrose and maltose, while specialist feeders are not. Among generalist feeders are herbivores and predators and specialist feeders include parasitoids and some mites (Singh, 1977; Cohen, 2004,2015). Predatory arthropods can obtain carbohydrates from prey in the form of the disaccharide trehalose and the polysaccharide glycogen which are the two main forms of carbohydrate reserves in insects; however, insect amylase can also digest starch (Chippendale, 1978), which is present in many nonprey food sources consumed by omnivorous predators.

8.2.2 Lipid

Lipids in the forms of fatty acids, sterols, and phospholipids are essential structural components of the cell membrane, they also provide an efficient way to store and provide metabolic energy during sustained demands, and they provide a barrier for water conservation in arthropod cuticle (Downer, 1978; Chapman, 1998).

Fatty Acids. In general, polyunsaturated fatty acids such as linoleic and linolenic acids are essential in insect nutrition. Insects are either unable to synthesize them altogether or incapable of synthesizing them in sufficient quantities. The inability of insects to synthesize polyunsaturated fatty acids has been confirmed in some species and limited capacity has been observed in other species such as mosquitoes, aphids, and cockroaches (Downer, 1978; Chapman, 1998). Derivatives of polyunsaturated fatty acids known as eicosanoids, stimulate oviposition in crickets and may be important for reproduction in all insects (Chapman, 1998).

Sterols. Insects are unable to synthesize sterols and therefore they are essential nutrients throughout Insecta (House, 1961; Chapman, 1998). Sterols play a variety of important roles in insect physiology as components of subcellular membranes, precursors of hormones, constituents of surface wax of cuticle, and constituents of lipoprotein carrier molecules (Downer, 1978; Chapman, 1998). Insects obtain sterols from cholesterol, but other important sources of sterols include plant phytosterols and ergosterol from fungi (Downer, 1978; Cohen, 2004, 2015).

Phospholipids. Insects synthesize phospholipids in the fat body (Downer, 1978). Phospholipids play an important role in lipid transfer and in the synthesis of vitellin and other lipoproteins (Agosin, 1978; Shapiro, 1988). Phospholipids are easily digested and absorbed by insects and are a good source of polyunsaturated fatty acids (Chapman, 1998).

8.2.3 Protein

Proteins are classified according to their solubility and function as globulins, nucleoproteins, lipoproteins, and insoluble proteins. Globulins include enzymes, antibodies, and protein hormones. Nucleoproteins are associated with nucleic acids and ribosomes. Lipoproteins often serve as transport proteins and insoluble proteins are passive compounds often referred as structural proteins (Agosin, 1978).

Amino Acid Profile. Arthropod diets should contain at least 10 amino acids considered essential which include leucine, isoleucine, valine, threonine, lysine, arginine, methionine, histidine, phenylalanine, and tryptophan (Chapman, 1998; Cohen, 2004, 2015; Lundgren, 2009a). These 10 amino acids are considered essential in insects due to their inability to synthesize them. Other amino acids that can be synthesized but only in insufficient quantities, or that require large consumption of energy for their synthesis may be also required in the insect diet (House, 1961; Chapman, 1998; Lundgren, 2009a). Tyrosine is a major component of sclerotin and is required in large quantities during molting (Hopkins, 1992). Proline is important during flight initiation by elevating sugar metabolism (Wigglesworth, 1972; Carter et al., 2006; Lundgren, 2009a), serine, cysteine, glycine, aspartic acid and glutamic acid are essential for silkworm growth (Chapman, 1998).

In recent years, assessment of the proportion of amino acids, lipids/fats, or glycogen in arthropods through a "carcass analysis" has provided useful information for comparing the nutritional value of food in relation to the arthropods consuming the food. For example, Lindig et al. (1981) conducted an analysis of essential and total amino acids in the carcasses of three insect pests (pecan weevil *Curculio caryae* (Horn), southwestern corn borer *Diatraea grandiosella* Dyar, and tarnished plant bug *Lygus lineolaris* (Palisot de Beauvois)). A comparative amino acid analysis of host plants of these insect herbivores (i.e., pecan kernels, inner whorls of corn, and pods of green bean) was also conducted. The authors concluded that the percentage of essential amino acids in each insect was comparable to that in each host. Lindig et al. (1981) surmised that the analysis of the insect carcass could provide information on the protein types to incorporate into an artificial diet for healthy development of the insect species under investigation. A broader application of this approach to include other nutrients such as sugars and lipids may prove valuable.

8.2.4 Vitamins

Vitamins are grouped into water and lipid-soluble based on their solubility properties. Vitamins are usually required for growth in insects, which in general are unable to synthesize them (Chapman, 1998).

Water-soluble vitamins include vitamin "C" and the "B" complex, which is comprised of thiamin (B_1), riboflavin (B_2), niacin = nicotinamide (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9), and cobalamins (B_{12}). The B vitamins function as cofactors for enzymes and are required in the diet of all insects (Chapman, 1998), except for vitamin B_{12}, which is not universally required (House, 1961; Cohen, 2004,2015). Predatory arthropods can obtain B complex vitamins from prey but nonprey foods such as fungi, which also contain high quantities of B vitamins

(Lundgren, 2009a). Vitamin C (ascorbic acid) is more important in herbivorous than in entomophagous insects, but it is known to play a role in the molting process (Chapman, 1998; Cohen, 2004, 2015). Vitamin C is also an antioxidant and may play an important role in the detoxification processes and protection against microbial infection (Cohen, 2004, 2015). Inositol and choline are constituents of some phospholipids. Choline plays a role in spermatogenesis and oogenesis in addition to its structural role in phospholipids and it is probably required in all insects. Inositol is known to be required in Coleoptera and plays a role in the nervous system (Chapman, 1998).

Lipid soluble vitamins include retinol, carotenoids (A), tocopherols (E), calciferol (D), and phyloquinone (K). Only vitamins A and E are known to be required in insects, where they play a role in the synthesis of pigments and in reproduction, respectively (Chapman, 1998). Both Vitamins A and E are also antioxidants (Cohen, 2004,2015). Fat-soluble vitamins are usually found in high quantities in nonprey foods like pollen and grains (Lundgren, 2009a).

8.2.5 Minerals

While some elements like nitrogen, sulfur, iron, and phosphorous can be obtained from organic sources other essential elements for growth and reproduction must be obtained from inorganic sources (minerals) which cannot be biosynthesized. Twenty four elements are known to be essential for living matter in order of importance: hydrogen, oxygen, carbon, nitrogen, calcium, phosphorous, chlorine, potassium, sulfur, sodium, magnesium, iron, copper, zinc, silicone, iodine, cobalt, manganese, molybdenum, fluorine, tin, chromium, selenium, and vanadium (Hammond, 1996). Elements can be divided according to their ionic charge into cations (+) and anions (−). Cations include metals like iron, sodium, potassium, magnesium, manganese, calcium, zinc, and copper. Anions include chloride, sulfur, fluoride, phosphorous, and iodine. Minerals are compounds that consist of combinations of cations and anions. House (1961) mentions potassium, sodium, phosphorus, magnesium, manganese, zinc, and copper as important in insect growth. Iron is an important electron exchange cofactor in many enzyme pathways including in the synthesis of DNA (Cohen, 2004,2015). Calcium is required in a lesser extent in arthropods than in vertebrates but still plays an important role in muscular excitation (Cohen, 2004,2015).

Rarely have nutrient or tissue levels of trace elements been determined for insects (Cohen, 2004, 2015; Nation, 2008). Hence, the concentrations of trace elements have seldom been evaluated for their impact on insect production. This is in sharp contrast to protein, lipid and carbohydrate levels. Yet, the nutritional and physiological importance of trace elements in insects has been well established (Dadd, 1985; Lavilla et al., 2010), as well as their role in regulating gene expression via transcription factors (Zinke et al., 2002). The primary reason for this gap in knowledge and assessment of trace elements has been the difficulty associated with the detection and accurate determination of concentrations. Recent advances with inductively coupled plasma mass spectrometry (ICPMS) have provided an excellent solution for the simultaneous determination of several trace elements over a range of low concentrations making it an ideal method for working with insects.

Information on the dynamics between the levels of trace elements in food and levels retained within the insect reared on the food has been a starting point for the application of ICPMS in determining trace element levels in predatory heteropteran (Coudron et al., 2012). Coudron et al. (2012) clearly showed that the levels of trace elements in the food stream affect the levels within the predator *P. maculiventris*, and those levels likely affect the health of the beneficial. Specifically, they demonstrated that trace element concentrations in nymphs were different than in adults and that the content of trace elements in both nymphs and adults differed greatly from the content in the food. Although the trace element content in eggs differed from the food there was no significant difference in the trace element content in eggs regardless of the food source for 9 of the 10 trace elements measured; demonstrating homeostasis of trace elements in eggs independent of the food source. This showed the ability of *P. maculiventris* to actively and selectively regulate absorption, sequestration and/or accumulation of each trace element and that the regulation varies with developmental stage. These results indicate that the bio-availability of trace elements in different food sources can be compared using these methods, which would be another piece of information seldom recorded previously. Additionally, trace elements have a unique characteristic of not being formed nor destroyed (different from proteins, lipids, and carbohydrates) but have an integral physiological role. These qualities may enable the use of trace elements to determine relationships between nutrition and fitness in ways that could make them excellent bio-markers as described above.

8.3 Determining the basic diet formulation

8.3.1 Chemical analysis

An underlying assumption of these studies is that the most optimal diet for an entomophagous insect would be identical to or very closely resemble the biochemical tissue composition of the natural prey on which they have evolved in the

field (with the addition of plant-sourced tissue components in the case of omnivorous species). This is complicated by the fact that many entomophagous insects consume multiple prey species. Factitious prey species on which predators develop adequately in the lab may also be assumed to have all necessary nutrients. Thus, a prelude to developing efficient artificial diets for predatory insects may involve an assessment of the biochemical makeup of the predator, its prey, and the host plant. An examination of the biochemical makeup of natural enemies has been conducted in just a few studies. For example, Specty et al. (2003) did a carcass analysis of the predatory lady beetle *Harmonia axyridis* Pallas and its prey aphid *Acyrthosiphon pisum* (Harris) or factitious prey *Ephestia kuehniella* Zeller eggs. They discovered that the biochemical composition of the carcass of the lady beetle varied according to food source. *H. axyridis* reared on aphids had lower body protein content than those reared on *E. kuehniella* eggs. Similarly, the lipid content of *H. axyridis* was lower in individuals reared on aphids than when reared on factitious food. In a companion study, Zapata et al. (2005) found that a biochemical analysis of carcasses of the predatory mirid bug *Dicyphus tamaninii* Wagner proved useful in reformulating an artificial diet for this species. Carcasses of individuals reared in artificial diet showed deficiencies in the content of essential fatty acids and amino acids as compared to individuals reared on their prey (Zapata et al., 2005).

A technique that determines the composition of key elements, such as carbon (C), nitrogen (N), and phosphorous (P), in the body of arthropods and their food has been termed ecological stoichiometry by some (Sterner and Elser, 2002) and nutrient stoichiometry by others (Schoo et al., 2009). According to stoichiometry theory, the relative ratios of C:N:P in the tissues of an organism should match that of its food source. Any apparent imbalances in any of the elements, especially N and P, in the natural enemy could suggest that its food source was inadequate in one or more of these elements. Model organisms have typically been plants but now plant feeders (herbivores) and their natural enemies (omnivores, carnivores) have been incorporated into stoichiometric analyses (Fagan and Denno, 2004; Raubenheimer et al., 2007). There have been no studies using stoichiometry to assess the elemental composition of natural enemies used in the biological control of crop pests. This technology should be useful in designing and refining artificial diets.

Artificial diets have been developed using information from chemical analysis of the host or prey which was used to approximate the nutritional requirements of its parasitoid or predator (Rojas et al., 1996, 2000; Fercovich et al., 1999; Reeve et al., 2003). The most suitable natural food source for the targeted entomophagous arthropod was used to develop chemically defined artificial diet formulations. These formulations were typically designed to replicate the concentration and ratios of the major nutritional component of the host or prey. This was intended to eliminate the process of trial and error by simultaneously providing all major dietary components in adequate concentrations. Chemical analyses are performed with high-performance liquid Chromatography (HPLC), gas chromatography (GC), and mass spectrometry (Ms). These methods of analysis are specially directed to determine the content, concentration, and ratios of amino acids, carbohydrates, lipids, protein, and vitamins found in the bodies of the host or prey. Quantitative analysis of the major nutritional components can be done by comparing mathematical integrations of instrument outputs of samples with outputs generated with standardized solutions of the target components. This method allows the determination of the specific instrument sensitivity for each component and provides a method to convert area units to parts per million (Rojas et al., 1996, 2000; Fercovich et al., 1999; Reeve et al., 2003).

Amino acids, salts, carbohydrates, water and fat-soluble vitamins can be analyzed by HPLC (Dionex D500 microbore system, Dionex Co). Amino acids are extracted with a 0.1 N HCl aqueous solution at a 1:10 ratio (sample: solvent). Fresh samples are transferred to a centrifuge tube containing traces of bezophenyl urea and centrifuged for 15 minutes at 14,000 rpm. The solution is filtered using a 22-μm nylon filter. About 20 μL of the sample is injected into the HPLC equipped with an AminoPac-PA10 column as directed by the manufacturer (Table 8.1).

Mineral salt, carbohydrate, and water-soluble vitamin samples are prepared in the same manner but using deionized milli- Q water as extraction solvent. These types of compounds can also be analyzed with Dionex D500 system using IonPac-AS11-HC column for anions (fluoride, chloride, nitrite, bromide, nitrate, phosphate, and sulfate), IonPac-CS-12A for cations (lithium, sodium, ammonium, potassium, magnesium, and calcium), CarboPac-PA10 for carbohydrates (glucosamine, galactose, glucose, fructose, maltose, and sucrose) and Waters Symmetry C18 for water-soluble vitamins (ascorbic acid, thiamine, pyridoxal chloride, niacinamide, PABA, folic acid, B12 and riboflavin) (Table 8.1). Fat-soluble vitamins can be extracted from 350 mg of the sample with 4 mL of 1% citric acid in 80% ethanol-water plus 2 mL hexane. The mixture is shaken and vortex mixed for 1 minute, and placed into an ultrasonic bath at 40°C for 5 minutes. The sample is then centrifuged at 10°C for 5 minutes. to allow the hexane layer to be separated. The hexane portion is washed 3 times in this manner and then concentrated by evaporation under a stream of nitrogen to a 1 mL volume (Dong and Pace, 1996). The Dionex HPLC system can be used to analyze samples using Acclaim 120 C18 5um column (Vitamin A acetate, Vitamin D and Vitamin E) (Table 8.1).

TABLE 8.1 Synopsis of the analytical methods for detailed chemical analysis of basic nutrients present in bean leaves.

Chemical groups	Solvent for extraction	Analytical instrument	Analytical column
Amino acids	0.1 N HCl	ICMB	AminoPac-PA10
Anions	Water	ICMB	IonPac-AS11-HC
Cations	Water	ICMB	Ion Pac-CS-12A
Carbohydrate	Water	ICMB	CarboPac-PA10
Water-soluble vitamins	Water	HPLC	Acclaim-C18
Fat-soluble vitamins	1% citric acid in 8% ethanol–water	HPLC	Acclaim-120 C18 5 μm
Soluble protein	0.9% NaCl solution	SPM	
Fatty acids	Methanol-chloroform (2:1)	GC/MS	HP-5% MS
Sterols	Methanol-chloroform (2:1)	GC/MS	HP-5% MS

ICMB, Ion chromatography micro bore; *HPLC*, high-performance liquid chromatography; *SPM*, spectrophotometry; *GC/Ms*, gas chromatography and mass spectrometry. Water = reverse osmosis (Milli-Q) or distilled.

Soluble protein is extracted from fresh material with 0.9% sodium chloride containing traces of sodium azide at 1:10 ratio into a 2 mL centrifuge tube by maceration and then centrifugation at 14 rpm for 15 minutes. A sample of the liquid is then reacted according to Bio-Rad (Detergent Compatible) protein assay protocol. A standard curve is calculated also following the protocol procedure. The percentage of soluble protein is measured in a UV-Vis Auto Spectrophotometer at 750 nm wavelength.

Fatty acids are extracted with a methanol-chloroform (2:1) solution to a 1:10 ratio sample solvent. Samples are dried in a vacuum oven at 40ǫC and 20 psi pressure for 24 hours before being extracted. Immersed samples are set at room temperature for 1 hour and liquid is then filtered through a glass puppet packed with glass wool. Samples are rinsed 3 times with equal amounts of extracting solvent, filtered and combined. The combined samples are dried under a stream of nitrogen on a hot plate. Dry samples are reacted to their methyl ester form following Alltech GC Boron trifluoride/Methanol procedure. Dry methyl esters are re-dissolved in 0.700 mL hexane for their analysis. Samples can be analyzed using an Agilent Technologies 6890 N GC with a 5975 inert XL Mass Selective Detector (MSD), an Agilent 19091S-433 HP-5Ms 5% Phenyl Methyl Siloxane capillary column (Table 8.1). Sterols are extracted with a methanol-chloroform (1:2) solution to a 1:10 ratio sample-solvent for 1 hour and filtered as described above. Residues must be washed 3 times with an equal volume of methanol-chloroform and distilled water making sure that layer separation is distinct. The bottom layer of each rinse is collected, combined in a vial containing sodium sulfate and dried on a hot plate under a stream of nitrogen. The samples are re-dissolved in extracting solvent and analyzed in a GCMS as described above.

8.3.2 Water content

The optimal water content of an artificial diet is approximately the same as the water content in the body of the host or prey (Cohen, 2004, 2015). The body water content may differ among organisms and must be determined for each host or prey species during the process of artificial diet development. Wigglesworth (1972) mentioned that water content in insect bodies may range from 50% to 90% of total body weight. For instance, the water content in *Tenebrio molitor* L., *Zophobas morio* F., *Galleria mellonella* L., *Bombyx mori* L. larvae, and *Acheta domesticus* L. nymphs is 61.9%, 57.9%, 58.5%, 82.7%, and 77.1%, respectively (Finke, 2002). One simple way to determine water content is by the differential of wet weight minus the dry weight. Water content is very important especially in artificial diets for parasitoids because their water needs must be completely fulfilled by the diet formulation (Thompson, 1980,1981; Rojas et al., 1996; Fercovich et al., 1999). In egg parasitoids, such as *Trichogramma* spp., water content also impacts osmotic pressure affecting the survival of parasitoid larvae (Grenier, 1994). Because predator arthropods drink water freely, the water content in predator artificial diets can be more flexible. Predators with extra-oral digestion such as phytoseiid mites, heteropterans, and chrysopids can process solid foods (Cohen and Urias, 1986; Cohen, 1998; Cohen and Urias, 1986; De Clercq and Degheele, 1992; Cohen and Smith, 1998).

8.3.3 Macronutrient ratios

The ratio of the three macronutrients, lipid, protein, and carbohydrate can be used as the basis for an artificial diet formulation. The macronutrient ratios from food items are calculated as $R_L = L/(L + P + C)$, $R_P = P/(L + P + C)$, and $R_C = C/(L + P + C)$, where L, P, and C are the contents in g/100 g of lipid, protein and carbohydrate, respectively. These ratios change in different arthropod species and nonprey food sources and can be characteristic of the basic nutrition of each species.

Detailed nutritional analyses of most commercially available insect species have been done including *T. molitor*, *Z. morio*, *G. mellonella, A. domesticus* (Finke, 2002), *B. mori* (Finke, 2002; Mishra et al., 2003), *Antheraea assamensis* Helfer (=*A. assama*) (Mishra et al., 2003), *A. pernyi* (Guérin-Méneville) (Zhou and Han, 2006), *Samia ricinii* (Boisduval) (Longvah et al., 2011), *Musca domestica* L. (Finke, 2013; Inaoka et al., 1999), *Hermetia illucens* L., *Chilicomadia moorei* Silva, *Blatta lateralis* Walker (Finke, 2013), and *A. kuehniella* (Specty et al., 2003). Lipid, protein and carbohydrate ratios of those commercially produced insects can be calculated from published data (Fig. 8.1) and utilized as the basis for a carnivore diet. The macronutrient ratio can be used to set the basic limits for these nutrients during the artificial diet development process. However, this method has a more important application during diet refining stages (Section 8.50.2) and for the substitution of chemically defined ingredients.

8.4 Presentation

Artificial diets can have a consistency ranging from liquid to solid depending mostly on two factors, water content and molecular cohesion. The first artificial diets were liquid (Thompson, 1980; House, 1978). The major disadvantage of liquid diets is that all nutritional components must be in solution or in stable suspension. Providing adequate sources of protein and lipids in liquid diets becomes difficult for some arthropod species (Rojas et al., 2000). Protein must be provided in soluble form (globulin) and lipids must be emulsified in water by using soaps. Because egg parasitoids typically develop in a semiliquid medium within the host egg, a successful artificial diet is likely to be presented as an encapsulated liquid aliquot (Grenier, 1994). The same often applies to endoparasitoids (koinobiont), which develop within the hemocoel of the living hosts (Quicke, 1997). Endoparasitoids have access to a continuous source of globulins and lipids in the form of phospholipids and glycoproteins inside the living host (Agosin, 1978; Chapman, 1998). Insect eggs contain vitellum, which is composed of glycoproteins and lipo-glycoproteins (vitellin). Lipo-glycoproteins consist of a combination of phospholipids, carbohydrates and proteins (Chen, 1978; Hagedorn and Kunkel, 1979). The complexity of such molecules allows the storage of large quantities of nutrients with little increase in osmotic pressure. Liquid diets for egg parasitoids must provide all nutrients in solution and at the same time maintain an optimal osmotic pressure to maintain parasitoid good health (Grenier, 1994).

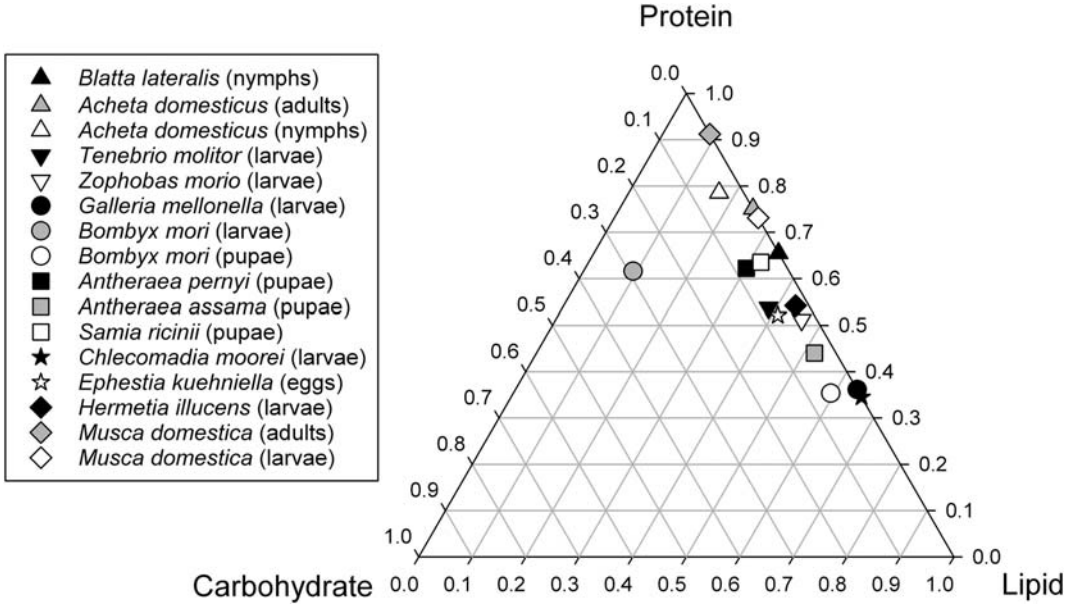

FIGURE 8.1 Basic nutrient ratios of lipid, protein, and carbohydrate of commercially produced insects. *Data adapted from Inaoka et al. (1999), Finke (2002, 2013), Mishra et al. (2003), Specty et al. (2003), Zhou and Han (2006), and Longvah et al. (2011).*

8.4.1 Feeding adaptations

Feeding adaptations determine the way food is processed by insects before ingestion and greatly influence the way artificial diets must be presented. Mouth parts in insects have been classified as mandibulate and hustellate (Borror et al., 1976). Most coleopteran and neuropteran adult predators have mandibulate mouth parts with well-developed chewing mandibles. Larvae of some Coleoptera and most larvae of Neuroptera possess modified mandibles with channels that enable them to suck digested liquids of their prey; this mandible type has been named grasping-sucking (Snodgrass, 1935). Hymenopteran parasitoids usually have a hymenopteriform larva with a poorly defined head capsule, but with well-defined functional mandibles (Gauld and Bolton, 1996). Heteropteran predators like the rest of the Heteroptera possess hustellate; highly modified mouth parts (Borror et al., 1976) specialized for sucking liquefied food following extraoral digestion (Cohen, 1990). Mite predators, such as members of Chelicerata, have chelicerae as mouth parts used in extraoral digestion. Chelicerae of phytoseiid mites can be articulated, chelate, and dentate pincerlike structures capable of cutting holes in the prey's body (Walter and Proctor, 1999).

8.4.2 Encapsulation of liquid diets

Unlike the more solid diets suitable for mandibulate chewing, liquid diets require some type of enclosure for containment and presentation, regardless of whether the entomophagous arthropod develops within or feeds through the containment. Liquid artificial diets have been encapsulated to mimic artificial eggs using different materials, such as paraffin, PVC, polyethylene or polypropylene; *Trichogramma* species are perhaps the most successful example of rearing egg parasitoids by this method (Grenier, 1994). House (1978) used Parafilm to encapsulate a semiliquid artificial diet for the endoparasitoid *Itoplectis conquisitor* (Say). Microliter volumes of liquid or semiliquid diets have been encapsulated in the wells formed with stretched Parafim to rear the predatory heteropteran *Perillus bioculatus* (F.) (Rojas et al., 2000), and domes formed with Mylar-Parafilm were used to contain a diet for *Podisus maculiventris* (Say) (Wittmeyer et al., 2001). Larger volume pouches were used to present a semiliquid diet for *Lygus hesperus* Knight (Patana, 1982) and *L. lineolaris* (Cohen, 2000). Because of its physical properties, Parafilm is a difficult material to sterilize, form, or stretch in a mechanized system. Some absorbent materials like polyester padding (Rojas et al., 1996) or cotton (Dindo et al., 2003) have been used to contain liquid diets for the pteromalid ectoparasitoid *Catolaccus grandis* (Burks) and the tachinid endoparasitoid *Exorista larvarum* (L.), respectively. Such materials can be easily cut to size and sterilized in an autoclave before being impregnated with the diet formulation. Other fabric materials made of synthetic fibers of high melting points such as nylon and polypropylene provide equally viable media for diet containment and presentation. Diets with semiliquid to semisolid consistencies resembling gel or paste can be presented sandwiched between two sheets of screens made of high fusion point synthetic materials (Rojas, unpublished). Screens provide a way for the arthropods to walk without getting trapped by the sticky diet, while at the same time providing easy access to the diet for feeding.

8.4.3 Gels and carriers for solid formulations

Traditionally, solid diets have been developed for insects with well-developed chewing mouth parts. However, Cohen (1990, 1998) showed that arthropods with extra-oral digestion can feed on solid diets, even if they possess sucking mouth parts with no functional mandibles. Entomophagous arthropods with extraoral digestion include spiders, phytoseiid mites, heteropterans, neuropterans, and some coleopteran larvae (Cohen, 1998). Larvae of the ectoparasitoid Hymenoptera are also fed by extraoral digestion (Cohen, 2004, 2015) and this was proven by successfully rearing *C. grandis* on solid diets (Rojas et al., 1996). Solid diet formulations for predatory insects have also been encapsulated using Parafilm to create a barrier simulating the host cuticle, for instance, Cohen and Urias (1986) rolled cylindrical shapes of diet and wrapped them with Parafilm to rear *Geocoris punctipes* (Say). De Clercq and Degheele (1992) used the same procedure and diet formulation to rear *Podisus sagitta* (F.) and *P. maculiventris*. A solid diet placed in cell culture wells sealed with Parafilm was used to rear *Chrysoperla rufilabris* Burmeister (Cohen and Smith, 1998).

Solid diets have advantages over liquid and semiliquid diets, including nonstickiness and in many cases direct presentation without the need for encapsulation or containment. However, solid diets can have a large range of variations in water content, for instance, agar-based diets can contain over 90% water (Rojas et al., 1996; Cohen, 2004, 2015). Gelling agents are commonly used to make a diet solid. Cohen (2004) provided an excellent review of the most commonly used gelling agents and commented that these agents are usually the most expensive ingredient in diets. Gelling and thickening agents have long been utilized in the food industry. For instance, alginates, kappa and iota carrageenan, gelatin, gellan gum, pectins, methyl cellulose, and mixtures of xanthan and locust bean gums function as gelling agents;

while gum Arabic, guar gum, carboxyl methyl cellulose, microcrystalline cellulose, and lambda carrageenan function as thickening agents (Fellows, 2009). Future directions on artificial diet development for entomophagous arthropods should include studies on the use of these agents to modify the consistency of the formulations into a more solid state that insects can ingest and digest.

8.4.4 Feeding stimulants

Nutritional deficiency can result from the lack of some key nutrients in a formulation, but also can be the result from low levels of consumption. Even artificial diets that match the nutritional characteristics of the natural host or prey may be perceived by the entomophagous arthropod as unrecognized or "unpalatable." Some artificial diet formulations may be consumed only after the onset of starvation and as the last resort to survive. Such formulations may still produce reproductive adults, but their quality may be substantially compromised. It is generally accepted that phagostimulation by chemical compounds is required to produce an excitatory effect for continuous feeding (Simpson, 1995; Chapman, 1995, 1998). Some secondary plant chemicals with no nutritional value have been identified as feeding stimulants in some herbivore insects as well as some nutrients like sucrose and inositol (Hanson, 1983). Compounds with no nutritional value which stimulate feeding have been termed token stimuli (Matthews and Matthews, 1978). Some organic acids resulting from the fungal decay of wood, such as oxalic acid (Espejo and Agosin, 1991; Green et al., 1995), stimulated feeding in the Formosan subterranean termite *Coptotermes formosanus* Shiraki (Morales-Ramos et al., 2009). In predatory insects, waxes present in the cuticle of prey may act as feeding stimulants (Greany and Hagen, 1981). Fatty acids present in *Tribolium castaneum* Jacquelin du Val induced feeding in the reduviid predator *Peregrinator biannulipes* Montrouzier and Signoret (Tebayashi et al., 2003). Some secondary plant chemicals are known to play a role in the prey finding sequence (Hagen, 1987), but little information exists on the phagostimulatory properties of those chemicals. The value of feeding stimulants to improve artificial diets has not been fully explored, but their potential has been recognized. For instance, feeding was stimulated and resulted in increased weight gain in the mirid mite-predator *Hyaliodes vitripennis* (Say) when β- sitosterol was added to a meridic artificial diet (Firlej et al., 2006). It is likely that feeding stimulants will play an important role in the improvement of artificial diets for entomophagous arthropods in the future.

8.5 Diet refining

Typically, after an artificial diet formulation has proven to be sufficient to allow the target arthropod to complete development and reproduce for more than five generations (level 2), the job of refining the artificial diet begins. Artificial diets that achieve level 2 criteria often produce arthropods of substantially lower quality than those produced in their natural host or prey. In most cases first or second-generation diet formulations (levels 1 and 2) tend to contain chemically defined components that are often too expensive to be used commercially. Additionally, successful artificial diet formulations are often susceptible to microbial contamination, which hinders their application in mass production systems. Because refining an artificial diet requires extensive and precise methods of evaluation and comparison, only diets that have reached level 3 are ready for the refining process due to the large number of in vitro-produced specimens that are required by the evaluation procedures.

8.5.1 Improving diet quality

Diet evaluation techniques. Rigorous metrics to assess the small, gradual improvements in the quality of insects reared on artificial diet formulations must be used. The high complexity of an artificial diet formulation makes it difficult to analyze the impact of small, iterative modifications made for improvement. The change in concentration of one single component automatically affects the concentration of the rest of the diet components. One way to begin the process of refining the diet formulation is to create several versions of the formulation with different modifications. These versions could have different macro-nutrient ratios or different concentrations of a particular set of nutrients. The most important aspect of this process is the method of evaluating the results of these diet variations on the biology of the in vitro-reared arthropod.

The biological parameters that are relevant for mass production include immature survival, development time, adult size, fecundity, egg viability, progeny sex ratio (in arrhenotokous species), and doubling population time (Cohen and Urias, 1986; De Clercq and Degheele, 1992; Rojas et al., 1996, 2000; Carpenter and Greany, 1998; Cohen and Smith, 1998; Morales-Ramos et al., 1998; Fercovich et al., 1999; Wittmeyer and Coudron, 2001). Behavioral parameters that are relevant for effectiveness as biological control agents include dispersal, host/prey finding, and host/prey handling capacities (Morales-Ramos et al., 1998). Evaluation of all the parameters mentioned above can be time-consuming,

especially for species with relatively long life cycles. Some biological parameters can be correlated to one another making the evaluations simpler to conduct (Grenier and De Clercq, 2003). For instance, Greenberg et al. (1995) reported that the pupal weight of *C. grandis* correlates with fecundity. However, that study was conducted in vivo and with only the natural host, *Anthonomus grandis* (Boheman), and no comparison was done among diet treatments (Greenberg et al., 1995). Changes in diet can have more profound effects on key physiological processes during the development of entomophagous arthropods impacting biological parameters independently of each other (Grenier and De Clercq, 2003). A good example of the independence of body weight and fecundity was mentioned by Grenier and De Clercq, (2003), where *P. maculiventris* reared on artificial diet were smaller than those reared on *T. molitor* larvae, but they had similar fecundities (De Clercq et al., 1998); in contrast with *P. bioculatus* reared on a diet that had the similar size to those reared on *Leptinotarsa decemlineata* (Say) eggs, but their fecundity was only one-tenth (Rojas et al., 2000).

Life history analysis by the use of life tables provides a means to determine the impact of multiple biological parameters on population growth. Carey (2001) categorizes life-history analyses by 3 types of life table methods (1) Lotka (2) field, and (3) classical life tables. Classical and field life tables focus on the analysis of age-specific mortality, but Lotka life tables use age-specific birth and survivorship to compute statistics associated with the Lotka stable population model (Lotka, 1907, 1928; Dublin and Lotka, 1925), such as net reproductive rate (*Ro*) and intrinsic rate of increase (r_m) (Carey, 2001). Immature survival, progeny sex ratio, development time, adult fecundity, and longevity can be all summarized using a Lotka life table in a single value defined as the intrinsic rate of increase (r_m) (Carey, 1993). This method is the most powerful tool of evaluation available and the most accurate and it provides a value (r_m) that is a measure of population fitness (Roff, 1992). Net reproductive rate (*Ro*) is defined as the average number of female offspring that would be born to a cohort of females and is calculated as

$$Ro = \sum_{x=1}^{k} lxmx$$

where *lx* is the proportion surviving at age "*x*," *mx* is female progeny produced per female of age "*x*," and "*k*" is the oldest age. Intrinsic rate of increase (r_m) is the rate of natural increase of a population that has reached stable age distribution and is calculated by the Euler−Lotka equation (Carey, 1993) as

$$1 = \sum_{x=1}^{k} e^{-rmx} lxmx$$

The exact value of r_m can be obtained by this equation using recursive iteration while changing the value of r_m in the equation until the results yield the value of 1. Doubling time (DT) defined as the time required for the population to double in numbers (Carey, 1993) can be calculated as:

$$\mathrm{Ln}(2)/rm$$

Another method to evaluate different versions of an artificial diet formulation is by comparing the efficiency of food utilization or the efficiency of food conversion into arthropod biomass. Analysis of consumption, assimilation, excretion, and conversion reveals how organisms respond to different foods and how food components affect growth (Scriber and Slansky, 1981). The most useful statistics are the efficiency of food conversion of digested food (ECD = B/(I-F)) and of ingested food (ECI = B/I), where B = biomass gained, I = food ingested, and F = food excreted (frass) (Waldbauer, 1968; Scriber and Slansky, 1981; Wiegert and Petersen, 1983). Food utilization analysis requires dry weight measurements, but wet weight measurements can be utilized if the approximate moisture content is known. Wet weight estimates are useful for live specimens. Mean moisture content of arthropod species can be predetermined as well as that of the food provided. Food utilization can be used for evaluating the impact of different diet formulations on the growth of immature arthropods. However, this method is not suitable for evaluating the impact of diets on fecundity. Nevertheless, food utilization is a relatively quick way to eliminate the less advantageous formulations reducing the number of diet variations to be compared using life table analysis.

The process of diet refining can be long and tedious due mostly to the time and labor required by the evaluation techniques available and may require investing several years of research. These difficulties often have been real obstacles to the development of artificial diets beyond level 3. Later in this chapter, we will discuss the possibility of substantially reducing the time and effort required to evaluate artificial diets by using proteomics and genomics as a method to determine gene expression. The use of "omics" technology to evaluate and compare artificial diet formulations requires the characterization of key molecular markers, which may be different in each species of arthropod.

8.5.2 From chemically defined to economically sound

Chemically defined ingredients such as amino acids, sterols, fatty acids, and vitamins add to the cost of artificial diet formulation. In order to develop an artificial diet formulation from level 3 to level 4, adequate substitutions must be found that make the formulation economically viable. For instance, the adequate content of fatty acids may be achieved using vegetable or fish oils (single or in combination) with a fatty acid profile similar to that of the chemically defined formulation. The fatty acid profile of the most common oils used as food is available online in the USDA nutritional database (USDA, 2010). The USDA database also provides a detailed content of the most important nutrients present in common food items including protein (amino acid profile), carbohydrate, lipid (fatty acid profile), vitamins, minerals, water, etc. Controlling the content of each of the most vital nutrients in a diet while making substitutions of chemically defined ingredients can be a difficult process. We propose a method consisting of 6 steps for diet refining: (1) determine the relative content of three macronutrient types, lipid, protein, and carbohydrate; (2) the amino acid profile of proteins (chemically defined additions may be necessary to address specific amino acid deficiencies); (3) the relative content of three types of lipids, saturated, monounsaturated, and polyunsaturated; (4) the relative content of three types of carbohydrates; sugars, digestible polysaccharides, and indigestible polysaccharides (fiber); (5) the final content of vital nutrients in the resulting diet formulation; and (6) the vitamin, mineral, and sterol deficiencies and water balance.

The relative content of lipid, protein, and carbohydrate provides a useful starting point for making substitution decisions. The content of these three macronutrient groups can be determined relatively easily (see above) from prey or host specimens and from artificial diet formulations. The ratios of lipid, protein, and carbohydrate from common foods are calculated as shown in Section 8.3.2. These ratios can be plotted in a ternary graph as shown in Fig. 8.2 for the ratios contained in food ingredients commonly used in insect artificial diets. This graphic representation provides visual information on the similarities of undefined food ingredients with the prey or host. Commercially mass-produced insects could be used as ingredients in artificial diets (Fig. 8.1) without compromising the basic principle of rearing a single species. Many commercially produced insects are processed and sold as dry powders. Such flours can be used as artificial diet ingredients. As the commercialization of insects becomes more prevalent, a larger variety of species may become available for future inclusion in artificial diets.

Amino acid profiles of protein can be calculated in a similar way as the macronutrient ratios by converting amino acid content in g/100 g into ratios based on the total amino acid content.

$$RAai = Aai \div \sum_{i=1}^{n} Aai$$

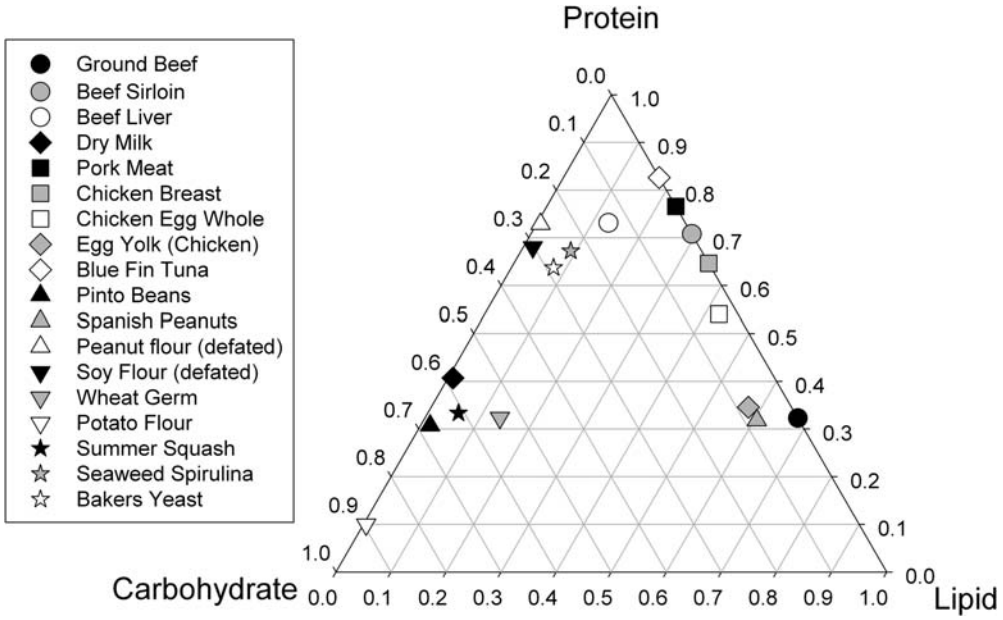

FIGURE 8.2 Basic nutrient ratios of lipid, protein and carbohydrate of food sources commonly used in insect artificial diets. *Data adapted from USDA, 2010. USDA national nutrient database for standard reference, release 22. <http://www.ars.usda.gov/Services/docs.htm?docid = 8964>.*

Amino acid content converted into ratio profiles can be compared independently of the relative protein content, which is established during step 1. Comparisons and adjustments are important only for the essential and conditional essential amino acids. Fig. 8.3 provides an example comparing amino acid profiles of four commercially produced insects, four proteins of animal origin, and four proteins of vegetable origin. Some amino acids are present in a higher proportion in insect protein than in vegetable or vertebrate protein, such as proline and tyrosine (Fig. 8.3C). Proline can be very important as a source of energy to initiate flight and omnivore predators obtain it from pollen feeding (Wigglesworth, 1972; Carter et al., 2006; Lundgren, 2009a). Tyrosine plays an important role in the process of sclerotization as precursors of proteins associated with cuticle tanning (Hopkins, 1992) and is probably required in all arthropods in higher quantities than in vertebrates. For this reason, meridic artificial diets may have to be supplemented with proline and tyrosine. Other amino acids may have to be added for some predators with prey choices containing unusual amino acid profiles or omnivore predators feeding in nonprey food with a unique profile of amino acids.

It is not necessary to create a full fatty acid profile for the substitution of lipids with chemically defined ingredients in step 3. Ratios of saturated, monounsaturated, and polyunsaturated fatty acids can be calculated using the same method used for the macronutrient ratios. These ratios can also be plotted in a ternary graph for better visual comparison (Fig. 8.4). Additionally, the actual content of essential polyunsaturated fatty acids such as linoleic and linolenic acids can be compared in step 5. Some vegetable (corn and soybean) and fish (salmon) oils provide high quantities of

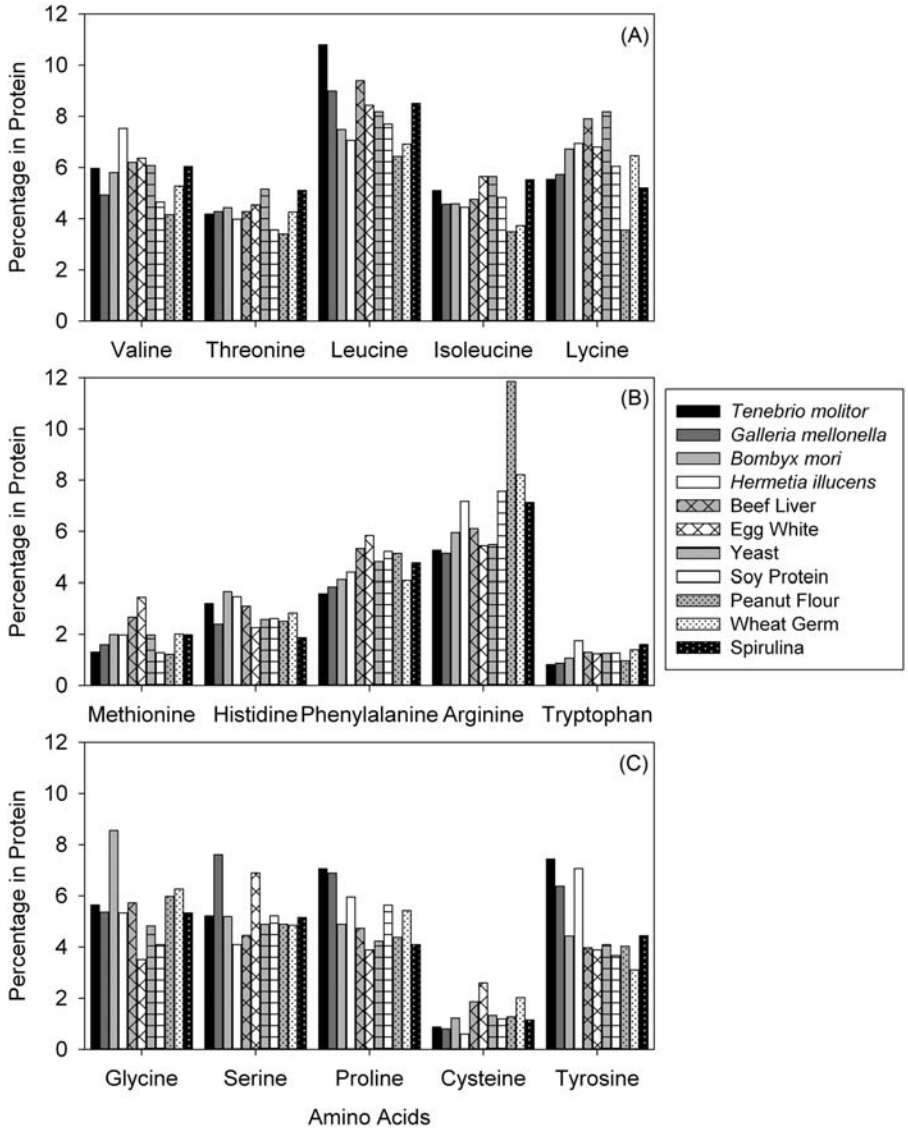

FIGURE 8.3 Amino acid ratios present in the protein of four commercially produced insects compared with two foods of animal origin and five foods of vegetable origin. (A) Simple molecule essential amino acids, (B) complex molecule essential amino acids, and (C) amino acids required in some species or hard to synthesize. *Insect data adapted from Finke (2002, 2013), food data adapted from USDA (2010).*

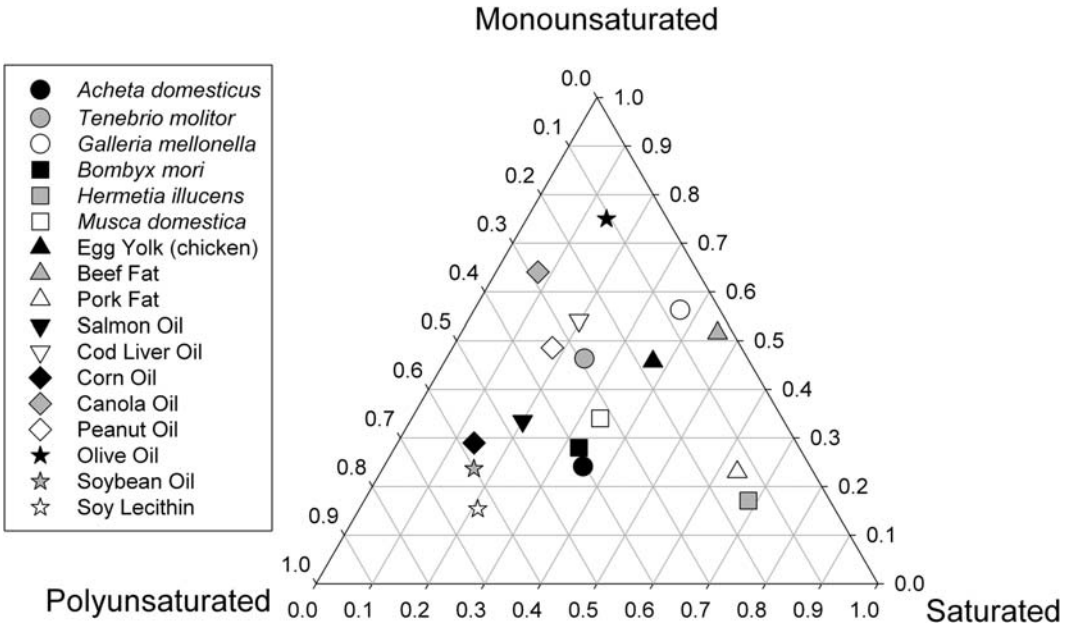

FIGURE 8.4 Ratios of three basic fatty acid types, saturated, monounsaturated, and polyunsaturated, present in the fat of six commercially produced insects compared to fats and oils of food products. *Insect data adapted from Finke (2002, 2013), food data adapted from USDA (2010).*

polyunsaturated fatty acids. Olive and canola oils provide high quantities of monounsaturated fatty acids (Fig. 8.4). Commercially produced insects have different ratios of the three types of fatty acids providing a diverse variety of choices, and useful for making decisions when choosing an insect as a protein source for the diet.

Carbohydrate substitution in step 4 is relatively simpler than protein and lipid substitutions. Because many chemically defined carbohydrates, such as sucrose, fructose, glucose, maltose, and starch are commercially available, adjusting sugar content in artificial diets can be done entirely by adding chemically defined ingredients. However, some food products such as honey, potato, corn starch, and powder milk can provide sufficient amounts of carbohydrates. The fiber component consists mostly of chitin, but can also include cellulose in omnivore predators, and can be substituted by cellulose powder. Fiber can be important in aiding the digestion of other food components even if the predators are unable to digest it.

Natural product substitutes for chemically defined components add many ancillary substances to the diet other than the intended components. It becomes necessary to keep track of these changes in order to maintain a correct balance of all nutrients as established by the original chemically defined artificial diet formulation. This can be accomplished in step 5 by a matrix operation (Fig. 8.5) where a vector "**v**" contains the proportions of each of the diet ingredients "*i*" (including the chemically defined). This vector is multiplied by a matrix "**M**" consisting of rows that represent the ingredients (eggs, powdered milk, beef liver, vegetable oil, yeast, etc.) and columns that represent the nutrients to be considered (protein, lipid, sugar, polysaccharide, vitamins, sterol, water, etc.). Elements of matrix "**M**" represent the content of each nutrient "*j*" in g/100 g for each of the diet ingredients "i". The nutrient content of the natural ingredients can usually be found in the USDA nutritional data base (USDA, 2010) as mentioned above. Matrix "**M**" needs to include only those nutrients critical for the development of our entomophagous species, but it can include as many nutrients as desired. These could include individual ingredients such as starch, glucose, proline, etc., and nutrient groups like lipid, protein, and carbohydrate. Multiplying vector "**v**" times matrix "**M**" results in a vector "**d**," which contains the same number of elements as a matrix row (Pielou, 1984) and represents the amounts in g/100 g of each nutrient contained in the new modified artificial diet (Fig. 8.5). Values of chemically defined ingredients in matrix "**M**" will be 100 for their corresponding nutrient and 0 for the rest of the nutrients.

The results from this matrix operation provides with an approximate content in g/100 g of all the critical ingredients in the diet formulation. This information can be utilized to determine and adjust the content of essential nutrients in the formulation (step 6). Because varying the content of one ingredient affects other ingredients, the diet vector "**d**" must be recalculated after every modification done to vector "**v**" and matrix "**M**."

Water content is a critical part of this analysis because many raw natural ingredients contain large amounts of water. This potential problem can be overcome by using ingredients in their dry presentation. Some basic natural ingredients such as beef liver, potato, milk, egg yolk and egg white are available as dry powders. Using dry ingredients provides

$$\mathbf{v} = (v_1, v_2, v_3, \ldots\ldots v_n),$$

Where elements are ingredient proportions with values between 0 and 1

$$\mathbf{M} = \begin{bmatrix} m_{11}\ m_{12}\ m_{13}\ m_{14} \cdots\cdots m_{1n} \\ m_{21}\ m_{22}\ m_{23}\ m_{24} \cdots\cdots m_{2n} \\ m_{31}\ m_{32}\ m_{33}\ m_{34} \cdots\cdots m_{3n} \\ m_{41}\ m_{42}\ m_{43}\ m_{44} \cdots\cdots m_{4n} \\ \cdots\cdots\ \cdots\cdots\ \cdots\cdots\ \cdots\cdots \cdots\cdots\cdots \\ \cdots\cdots\ \cdots\cdots\ \cdots\cdots\ \cdots\cdots \cdots\cdots\cdots \\ m_{n1}\ m_{n2}\ m_{n3}\ m_{n4} \cdots\cdots m_{nn} \end{bmatrix}$$

Where elements "m_{ij}" are g / 100g with values between 0 and 100, sub indexes 'i' and 'j' represent ingredients and nutrients, respectively.

$$\mathbf{vM} = \mathbf{d} = (d_1, d_2, d_3, d_4, \ldots\ldots\ldots d_n)$$

Where $d_j = \sum_{i=1}^{n} v_i m_{ij}$ for j = 1 to n with final values in g / 100 g

FIGURE 8.5 Method for calculating the total nutrient content of artificial diet formulations by multiplying the diet ingredient vector "v" by the food matrix "M." The diet ingredient vector consists of ingredient ratios (0−1). The food matrix consists of the nutrient content in g/100 g of key nutrients (columns) contained in the artificial diet ingredients (rows).

much more flexibility when adjusting the water balance of artificial diet formulations, but even dry ingredients contain a small amount of water that should be included in the matrix operation. After steps 1−6 have been completed, the result is a meridic formulation that closely resembles the initial chemically defined formulation. At this point, the new diet is ready to be evaluated using the methods described earlier in this chapter.

8.5.3 Industrialized insect components

In recent years, the industry of insect production for feed and food has increased exponentially (Dossey et al., 2016; van Huis, 2020). Insect species that are currently produced commercially include *Tenebrio molitor* L., *Hermetia illucens* L., *Acheta domesticus* L., *Zophobas morio* Fab., *Alphitobius diaperinus* Panzer, *Gryllodes sagillatus* (Walker), *Musca domestica* L., *Gryllus assimilis* (Fab.), and *Gryllus bimaculatus* De Geer (Cortes Ortiz et al., 2016; van Huis, 2020). Other species are available from the silk production industry including *Bombyx mori* L., *Antheraea pernyi* (Guénerin-Méneville), and *Samia cynthia* (Drury). Most of these insects are processed to powders that are now commercially available and constitute an addition to the list of ingredients available to formulate insect diets. Historically, artificial diet development was focused on the formulation of diets devoid of insect components. This was to avoid the necessity of rear additional insect species to obtain these components. However, with the availability of insect powders in the market, this limitation is no longer applicable.

The use of insect powders as a source of protein for insect artificial diet is preferable to the use of powders from the vertebrate origin such as liver powders from pork, beef, or veal. This is because the amino acid profile of insect protein fit closer with the requirements of insect predators or parasitoids than the protein of vertebrate or vegetable origin. Particularly amino acids associated with flight energetics like proline (Wigglesworth, 1972; Carter et al., 2006; Lundgren, 2009a) and amino acids associated with cuticle sclerotization like tyrosine (Hopkins, 1992). An example of the value of industrialized insect components in artificial diets for insect predators was provided by the successful improvement of a diet for *Coleomegilla maculata* De Geer (Rojas et al., 2016). The addition of 7% powder of *T. molitor* pupae to a meridic diet formulation without insect components showed significant improvements in immature survival, fecundity and egg viability in *C. maculata* (Rojas et al., 2016). This diet formulation was even superior to a control consisting of *E. kuehniella* eggs and bee pollen mix (Rojas et al., 2016). Substituting the *T. molitor* powder in the diet formulation for powders from other insect species provided additional improvements. Using powder from *Musca domestica* larvae or pupae in the diet formulation provided significant improvements in fecundity and progeny survival in *C. maculata* as compared with the diet with *T. molitor* larvae or pupae powder (Morales-Ramos, unpublished).

Hemolymph from industrialized insects has been used to improve artificial diets of fly parasitoids. In a study to improve the artificial diet of *Exorista larvarum* (L.), hemolymph from *H. illuscens* and *A. pernyi* were added to the formulation with mixed results (Dindo et al., 2016). While the addition of *H. illuscens* hemolymph to the diet produced significant improvements, the addition of *A. pernyi* hemolymph was detrimental (Dindo et al., 2016). Both examples cited above show that the species of industrialized insects used in diets have a significant impact on the efficacy of the diets. This is most likely due to the differences in nutrient content among different insect species (Finke, 2002, 2013), which will need to be customized for the particular insect to be reared in vitro. Industrialized insects are a promising source of ingredients for artificial diet development for insect predators and parasitoids.

8.5.4 Dietary self-selection

The ability of some insects to regulate the intake of nutrients has been well documented and has been referred to as dietary self-selection (Waldbauer and Friedman, 1991). The term dietary self-selection specifically refers to the consumption of different types of food items in an effort to achieve a specific balance of nutrients required by an organism (Waldbauer and Friedman, 1991; Behmer, 2009). Nutrient regulation by dietary self-selection has been demonstrated experimentally in a variety of insects including *Supella longipalpa* F. (Cohen et al., 1987), *Locusta migratoria* L. (Raubenheimer and Simpson, 1993; Trumper and Simpson, 1993), *Spodoptera littoralis* (Boisduval) (Lee et al., 2002), *Spodoptera exempta* (Walker) (Lee et al., 2004), *Helicoverpa zea* (Boddie) (Waldbauer et al., 1984; Schiff et al., 1988), *Helicoverpa virescens* F. (Lee et al., 2006), *Anastrepha obliqua* (Macquart) (Cresoni-Pereira and Zucoloto, 2001), *Tribolium confusum* Jacquelin du Val (Waldbauer and Bhattacharya, 1973), and *T. molitor* (Morales-Ramos et al., 2011). Dietary self-selection has also been demonstrated in some predatory species including *H. axyridis* (Coccinellidae) (Soares et al., 2004), *Agonum dorsale* (Pontoppidan) (Carabidae) (Mayntz et al., 2005), and *Ectatoma ruidum* Roger (Formicidae) (Cook and Behmer, 2010), and the wolf spider *Pardosa prativaga* (Koch) (Mayntz et al., 2005).

Most dietary self-selection studies have shown that insects regulate their food choices such that a species-specific balance of digestible carbohydrate and protein can be attained. For instance, *T. confusum* feeding on a three-component diet mix developed faster than when feeding on pure diets of each of the individual components (Waldbauer and Bhattacharya, 1973). Last instars of *H. zea* self-selected an 80:20 protein to carbohydrate ratio when offered two diets of identical and complete nutritional content except that one lacked casein and the other lacked glucose. The self-selected ratio supported the best food utilization and food conversion when compared to each of the individual diets and a diet with a 1:1 casein to glucose ratio (Waldbauer et al., 1984).

In some cases, diets self-selected by immature stages can benefit the adult stages as well. For instance, self-selected diet ratios by *T. molitor* larvae significantly increased adult fecundity and longevity (Morales-Ramos et al., 2013). Self-selected diets can also be stage-specific. For example, *S. longipalpa* developed better on a two-component diet of casein and glucose when allowed to self-select the proportions of a two-component diet rather than on either component singularly or a diet with 1:1 casein and glucose. However, when forced to feed on a diet with the same component ratio as the self-selected diet, the development rate was slower than the 1:1 ratio diet. The consumption ratio of the two components changed from high carbohydrate at the beginning to an equal ratio with protein by the end of the stadium (Cohen et al., 1987).

Insects feeding on diverse diets also self-select to balance nutrients other than carbohydrate and protein (Waldbauer and Friedman, 1991; Behmer, 2009). Dietary self-selection for lipids and vitamins has been documented in *H. zea*. Schiff et al. (1988) reported that last instar larvae of *H. zea* self-selected a superior mix of two incomplete diets. This experiment was similar to the one by Waldbauer et al. (1984), except that the lipid and vitamin content were changed in the two diets. Trumper and Simpson (1993) demonstrated that salt intake was regulated by self-selection in nymphs of *L. migratoria* when using incomplete artificial diets. However, regulation of protein and carbohydrate was stronger since nymphs of *L. migratoria* were driven to consume a 12-fold suboptimal salt intake in order to balance protein and carbohydrate intake in a two-choice experiment with incomplete diets (Trumper and Simpson, 1993).

Self-selection is particularly important in omnivore species, which feed on a variety of food sources. Many of the most important and commercially produced arthropod predators are omnivores at least to some degree (Eubanks and Styrsky, 2005). Nonprey food is not only a way to survive in the absence of prey; for most omnivore predators nonprey foods are important sources of energy for flight and play a role in reproduction and fecundity (Lundgren, 2009b). For instance, during the nonpredatory adult stage the syrphid predator *Rhingia campestris* Meigen consumes different ratios of pollen and nectar depending on the ovarial developmental stage (Haslett, 1989). Another important clue that indicates the importance of nonprey foods in reproduction is the fact that different sexes consume different proportions of nonprey foods (Lundgren, 2009b). One example is *Coleomegilla maculata* DeGeer where females consume 10 times more corn pollen than males (Lundgren et al., 2005).

It is reasonable to assume that consumption of nonprey food is not just supplementary, but it plays an important role in supplying all the nutritional requirements of omnivore predators. Consequently, omnivore predators must have the ability to adjust their nutritional needs by self-selection when confronted with a high diversity of food choices which have different nutritional characteristics. Soares et al. (2004) demonstrated that the coccinellid predator *H. axyridis* self-selected optimal ratios of two aphid prey species, *Aphis fabae* Scopoli and *Myzus persicae* Sulzer by showing that females allowed to self-select produced significantly more progeny than females fed with either of the 2 prey species alone. Mayntz et al. (2005) took a different approach to demonstrate self-selection capabilities in the carabid predator *A. dorsale* by offering adults a choice between lipid-rich and protein-rich diets comprised of powdered locust. Another approach consisted of allowing the wolf spider *P. prativaga* to choose between live prey of fruit flies (*D. melanogaster*) fed with lipid versus protein-rich diets (Mayntz et al., 2005). In both of the last two cases, predators showed the capacity to adjust their intake of protein and lipid by choosing among the two types of food (Mayntz et al., 2005). However, no studies have been reported on self-selection of prey versus nonprey foods in omnivore predators.

Dietary self-selection has not been extensively used as the basis for artificial diet development or refinement. However, Waldbauer et al. (1984) obtained a better diet formulation for *H. zea* after adjusting the casein-to-glucose ratios to those self-selected by the larvae. This achievement prompted Cohen (2004) to suggest dietary self-selection as a potential way to refine artificial diets. Morales-Ramos et al. (2013) were able to refine a dietary supplement for *T. molitor* using self-selected ratios of six components by groups of larvae. Adult *T. molitor* females fed with a supplement formulation derived from self-selected ratios by larvae produced significantly more progeny than females fed with other supplements or with no supplement (Morales-Ramos et al., 2013). In two more elaborated studies self-selection was used to determine the optimal number of undefined ingredients and their ratios to formulate improved diets for the house cricket, *A. domesticus* (Morales-Ramos et al., 2020a), and for *T. molitor* (Morales-Ramos et al., 2020b). In these two studies, the insects were capable of selecting optimal macro-nutrient ratios by choosing among complex undefined ingredients consisting of food products (potato, cabbage, corn meal, buckwheat, etc.) and agricultural by products such as dry distillers grains, defatted grain meals from vegetable oil production, and brans from grains like wheat and rice (Morales-Ramos et al., 2020a,b). These results demonstrate the great promise of this method in the development of oligidic diets for omnivore arthropods. In theory, self-selection should be applicable to omnivore predators, not only to adjust the components of existing diets but also to develop new diets starting with undefined components, which could include powders from industrialized insects.

8.5.5 Diet preservation

Artificial diets for entomophagous arthropods contain a balanced set of nutrients ideal for the growth of contaminant microbial agents such as bacteria and fungi. The introduction of contaminating microbiota from the insectary environment is often unavoidable. Diet preparation and dispensing, reuse of containers, nonsterile insect eggs, human handling and dust are common sources of contamination. Even small levels of contamination can substantially compromise the nutritional value of the artificial diet by depleting essential nutrients and altering other properties of the diet such as moisture levels, color, and volatiles, any of which may impact production by inhibiting insect feeding and growth or mating. In addition, metabolic byproducts produced by the microbial contaminants are often toxic for the arthropods and hazardous to insectary workers. Cohen (2004) listed 17 species of bacteria and 9 species of fungi commonly found in insect artificial diets. In the same publication, Cohen (2004) presents an excellent list of chemicals that have been utilized to control microbial growth in insect artificial diets. While preservatives such as methyl paraben, propionic acid, sodium benzoate, and formalin have been effective in herbivore diets (Debolt, 1982; Funke, 1983), entomophagous arthropods are often intolerant of those chemicals (Rojas unpublished).

8.6 Future perspectives

8.6.1 Multiple diet component testing

Artificial insect diets can contain nutrients, preservatives, gelling agents, stabilizers, emulsifiers, and natural food material. To optimize diet formulations, often diet components are changed empirically, one factor at a time, and insect performance is evaluated after each change using one or more performance parameters. Testing one diet ingredient at a time requires several factorial experiments that are time-consuming and may be limited in effectiveness (Niedz and Evens, 2016). This is because changing the amount of one factor is confounded with changes in the proportions of other ingredients and interactions among ingredients in a mixture.

A multivariate geometric approach that applies response surface models in combination with *n*-dimensional mixture design allows for identifying functional and interactive relationships between several diet components and multiple response parameters of interest (e.g., insect life-history parameters, diet evaluation criteria), and thereby enabling optimum diet blends to be achieved (Lapointe and Evens, 2008; Huynh et al., 2017). Unfortunately, there isn't an example of applying this method to diet formulations for an entomophagous insect. So, we present recent examples of the multivariate geometric approach combined with *n*-dimensional mixture design applied to artificial diet development for the northern corn rootworm and formulation improvements for the western corn rootworm (Huynh et al., 2019b,c). In this example, the input variables (X_{j1}, $j1 = 1...k_1$) were diet ingredients (e.g., wheat germ, casein, sucrose) that are used to make different diet formulations. The response variables of interest (Y_{i1}, $i1 = 1...n_1$) can be insect performance parameters (e.g., insect weight, survival, development) when insects are reared on these diets or diet evaluation criteria (e.g., diet contamination, shelf-life, cost of diet production). Response surface models $Y_{i1} = f(X_1, X_2, X_3,..., X_k)$ ($i_1 = 1...n_1$) where function $f(s)$ are usually low-order polynomials, are utilized to describe the relationships between insect performance parameters and diet ingredients. Identifying and fitting empirical models enable determination of principal diet components (X_{j2}, $j_2 = 1...k_2$, $k_2 \leq k_1$) responsible for improving the desirable parameters of interest (Y_{i2}, $i_2 = 1...n_2$, $n_2 \leq n_1$) and identifying diet components that have negative or minor effects on the responses measured. It is noteworthy that diet formulations should be treated as mixtures, and proportions of diet components for each formulation tested always sum to 1 ($X_1 + X_2 + ... + X_k = 1$, k is the number of diet ingredients tested). In the diet mixture experiments, the amount chosen for ingredients or components of a diet mixture, properly referred to as its proportion, cannot be varied independently because the proportions of all components must sum to 1. Scheffé (1958) developed mathematical models specifically for mixture designs, commonly referred to as Scheffé models or Scheffé polynomials, and the corresponding computations are simplified by modern software for design of experiments.

The geometry design space of a diet mixture experiment is necessarily of $n-1$ dimensions where n is the number of diet components tested. A diet based on a single ingredient has zero dimensions because the mixture cannot be varied. A two-component blend has a single dimension that can be envisioned as a line with the end points corresponding to pure blends of the individual components. Three-component and four-component blends can be represented by a two-dimensional triangle using a ternary plot and three-dimensional tetrahedron, respectively to represent all possible blend combinations.

The polynomial models are often displayed using Trace plots (e.g., Cox plot) which visualize the effects between all diet components tested and each insect performance parameter (Fig. 8.6). These plots depict the effect of increasing the proportion of one component in relation to a reference blend, while the relative proportions of all the other diet components are kept constant. In the trace plots, the slope indicates the direction and magnitude of the impact of each ingredient on all measured responses or vice versa, which points out the principal and unnecessary diet components that drive or have negative effects on the insect performance parameters.

The relationship between several diet components and one or more insect performance parameters is also explored and characterized using response surface plots (surface and contour plots) generated from the polynomial models (Fig. 8.7). The surface and contour plots, which are three-dimensional and two-dimensional diagrams, respectively that

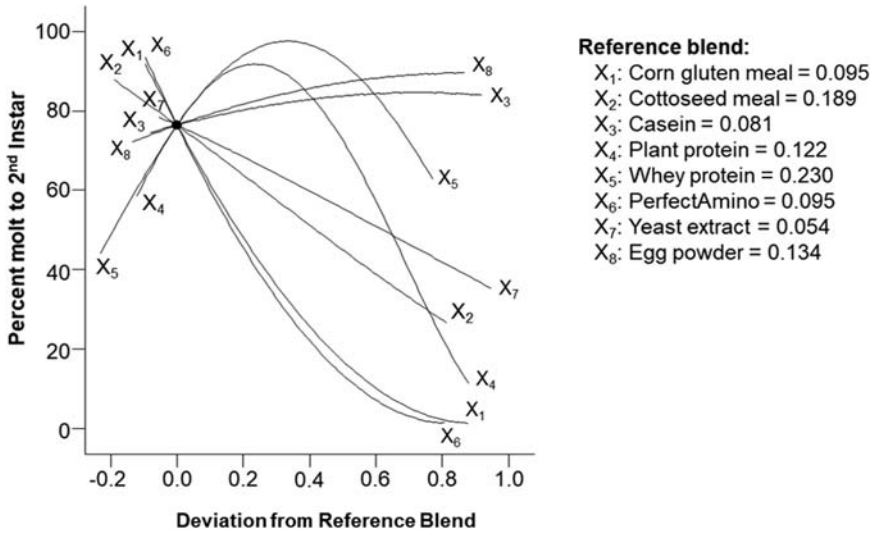

FIGURE 8.6 Cox plot describing the relationship between 8 diet components and percent molt of the northern corn rootworm in a diet mixture experiment. The slope of the line indicates the direction and magnitude of the influence of the ingredient on larval molt. The positive slope indicates that the associated diet ingredient has positive effects on larval molt, whereas negative slopes reveal negative effects of the diet ingredients on larval molt. *Data adapted from (Huynh et al., 2019b).*

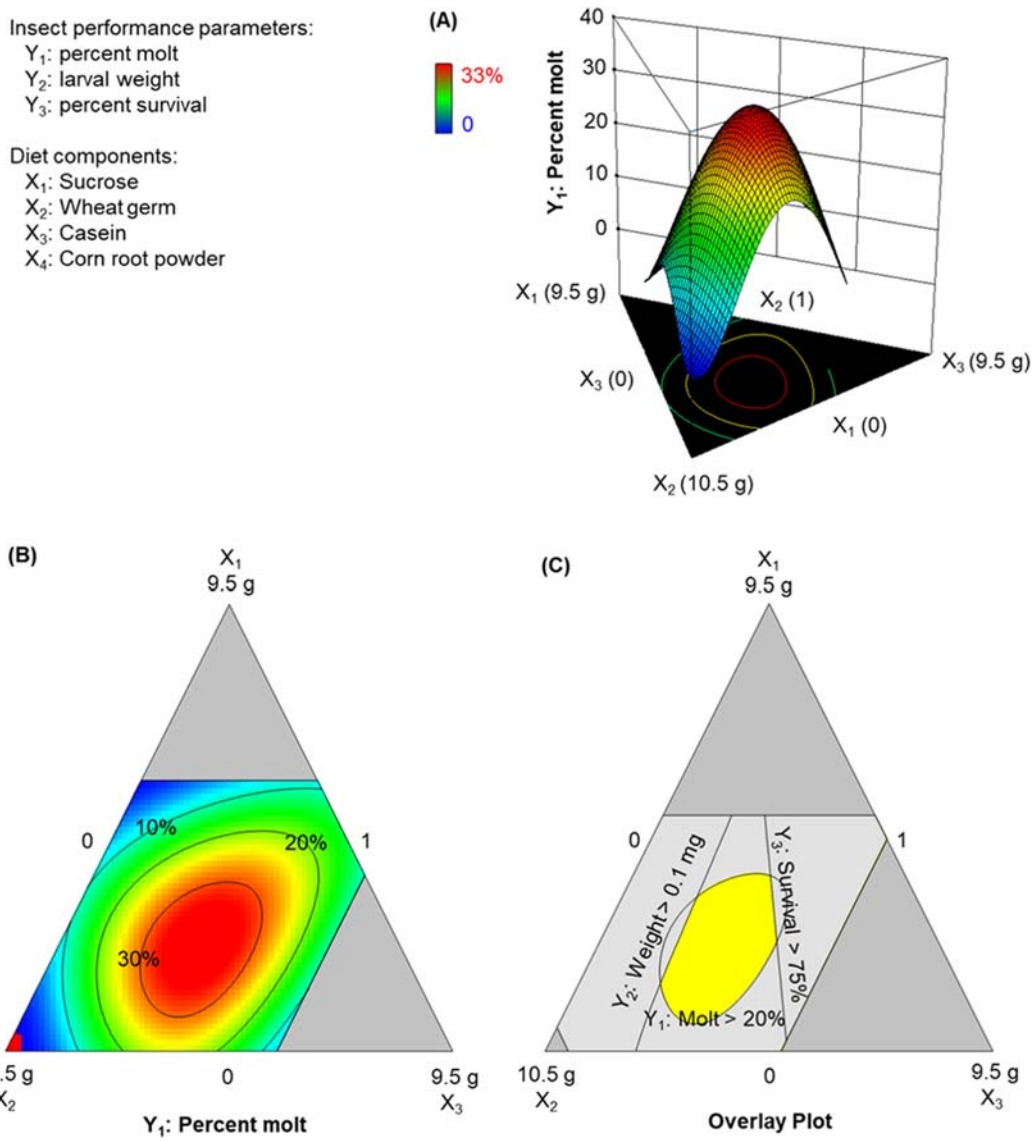

FIGURE 8.7 Response surface (A), its associated contour (B) and overlaid optimization (C) plots of a 4-mixture diet experiment. In these plots, X_4: corn root powder is kept constant. In the overlay plot, yellow area represents the optimum region which are approximated from an overlay of critical response contours for molt, weight, survival (Y_1, Y_2, Y_3) that are obtained when all four ingredients (X_1, X_2, X_3, X_4) are used. *Data adapted from (Huynh et al., 2017).*

describe functional relationships between each response parameter as a dependent variable (Y-axis) and two or three diet components (X_1, X_2, and X_3) as input variables. In order to identify the optimum diet blends, an overlaid optimization plot can be utilized to integrate all response parameters by overlaying their response surface plots on the geometry design space of diet components (Fig. 8.7). Noticeably, since the visualization is limited to three-dimensional, the response surface plots typically visualize the data of mixture experiments with two or three designed diet components, whereas other diet components are kept constant at the relative proportions.

The applications of the multidimensional approach for insect diet development typically include sequentially iterative steps (Huynh et al., 2019c). The first step is to identify a starting media, usually a current version of a diet needed to be improved or a diet of closely related species. The next step focuses on identifying principal diet components that can be in the initial media or new diet ingredients driving the insect performance parameters as well as removing unnecessary ones from the diet formulations. Follow-up mixture experiments are carried out to further characterize and optimize the key diet components to identify optimum diet blends that maximize or optimize the desired parameters. A final stage is usually a confirmatory experiment designed to validate desired goals of the process that can be achieved by the identified optimum diet formulations.

The use of the multidimensional approach to develop effective artificial diets for the northern and western rootworm provide a compelling example of the value of the technique for insect diet development. By applying the multidimensional approach, an improved and accessible diet formulation without the host plant material and with all available ingredients was achieved (Huynh et al., 2019a,b; Meihls et al., 2018) and the diet is now publicly and commercially available via the cooperation of USDA-ARS (Columbia, MO), University of Missouri (Columbia, MO) and Frontier Agriculture Services (Newark, Delaware).

8.6.2 Multiomic assessment of diet quality

Quality of food is broadly accepted as affecting the fitness and performance of beneficial insects, yet the association of nutrient quality with fitness (e.g., development, fecundity, etc.) is poorly understood. Most often studies have been limited to the effect of diet on biological parameters, for example, rate of development, weight and fecundity, and performance parameters important to biocontrol agents, for example, searching for and consuming prey. The coarseness and delayed timing of those measurements make it difficult to correlate differences in development or performance with specific dietary ingredients. This has undoubtedly resulted in overlooking critical dietary components and limiting the ability to optimize dietary formulations. Reoccurring instances where empirically formulated diets required the addition of small amounts of natural food to equal prey-fed performance and with the missing ingredient(s) remaining unidentified are excellent examples of these phenomena. Delayed measurement of nutritional quality as measured via biological and performance parameters can prevent early detection and correction of nutritional deficiencies, especially during highly sensitive growth stages. While these concerns are easily stated they have been difficult to remedy.

Recognizing that biological and performance functions are controlled by physiological functions, efforts have been made to measure the response of cellular functions to dietary changes, hoping to provide the sensitive and early indicators of interest. Unfortunately, using biochemical measurements to detect cellular responses to dietary changes has had limited success. This is probably because many of the biochemical substances measured were likely to be under homeostatic control, for example, vitellogenin. Hence, these substances demonstrated minimal changes or were slow to change in response to minor dietary alterations, rendering meaningful measurements difficult to reliably detect (Coudron et al., 2006). Several examples will be given below to suggest specific substances (like transcription factors) may serve as more sensitive and early indicators.

Using the parameter of differential gene expression for diet optimization, that is, nutrigenomics is a direct method to interrogate phenotypes, and hence promising as a way to accelerate diet development and identify dietary deficiencies (Yocum et al., 2006). Monitoring global gene expression (via RNA or protein) provides an opportunity to acquire information about the impact of nutrition on a wide range of biochemical and behavioral parameters. Essentially, nutrients act as dietary signals that are detected by cellular sensors, influencing gene, protein and ultimately metabolite levels (Müller and Kersten, 2003). This makes the insect the indicator of diet improvement by directly measuring gene expression that underlies favorable phenotypic changes. Potentially, a select set of those genes could serve as early and sensitive indicators that can be used to guide factitious host selection(s) or diet formulation processes in ways researchers have not yet been able to approach (Coudron et al., 2006; Yocum et al., 2006). This approach is a more data-rich, thorough assessment of the insect response to dietary changes. Additionally, this analysis is directed by the insect response rather than testing a predetermined substance for suitability as an indicator. That is to say, the results identify a marker rather than testing a known substance for its suitability to serve as a marker. Admittedly, the challenge is to "translate" those findings into meaningful markers to assist in selection of dietary ingredients. Yet, there is merit in exploring gene expression as a mean to optimize dietary selections.

The use of marker-assisted selection of nutrients for insects is in its infancy, but the concept was initially tested with artificial diets for the beneficial heteropteran *P. bioculatus*, the two spotted stink bug (Coudron et al., 2006). Of approximately 6000 genes surveyed in a cDNA microarray, 47 genes displayed a greater than 1.5-fold difference between artificial diet- and prey-fed third instar nymphs: giving an extensive set of genes for study. Of those, 31 genes were up-regulated in the diet−fed nymphs. Additionally, 61% of the genes were distributed in the biological functional class of cellular processes and 55% were categorized in the biological functional class of physiological processes (Coudron et al., 2006). From over 90 gene fragment sequences, four transcripts were selected for an investigation that had repeatable and significant differences between diet- and prey-fed nymphs (Yocum et al., 2006). Two gene transcripts up-regulated in diet-fed *P. bioculatus* nymphs compared to prey-fed nymphs had sequences similar to the tyrosine hydroxylase (TH) gene from *Apis mellifera* L. and the chitin-binding protein, *Gasp*, from *Drosophila melanogaster* Maigen. The sequence of two transcripts that were up-regulated in prey−fed nymphs did not match any gene of known function; an example of how a good biomarker may be of an unknown substance. TH catalyzes the rate-limiting step in

the biosynthesis of catecholamines (e.g., neurotransmitters) and impacts most biochemical processes, including ecdysis, behavior, immune response and reproduction. The *Gasp* precursor is a peritrophin-like protein thought to be associated with the peritrophins, a family of chitin-binding proteins associated with the peritrophic matrix of insects.

The TH and *Gasp* genes are of particular interest because they meet several of the criteria of ideal diet biological markers, that is, indicate a response to nutritional quality, have a high level of expression for ease of detection and occur early in development. A positive correlation was also found between levels of expression of these two genes and the number of generations the insects had been reared on the artificial diet (Yocum, et al., 2006). Two of the diet-fed up-regulated TH genes were tested for their responsiveness to nutrient alterations (Fig. 8.8). In one test diet-fed individuals were switched to prey which resulted in the disappearance of the up-regulated TH genes; demonstrating that the artificial diet was the cause of the up-regulation. In another test, the liver was removed from the artificial diet which again resulted in the disappearance of the up-regulated TH genes, demonstrating that the liver was the ingredient within the artificial diet responsible for up-regulating the TH genes. These tests indicate how gene or protein biomarkers can be used to evaluate nutrient components in diets.

Recent adoption of this nutrigenomics approach enabled the improvement of an artificial diet for *Arma chinensis* (Fallou) (Hemiptera: Pentatomidae) (Zou et al., 2013a, 2015, 2019). This is a predaceous insect species that effectively suppresses a wide range of agricultural and forest insect pests in the orders Lepidoptera, Coleoptera, Hymenoptera and Hemiptera (Fig. 8.9).

An insect-free artificial diet comprised of pig liver, chicken egg, and tuna was developed for *A. chinensis* (Zou et al., 2013b). That artificial diet was suboptimal compared to pupae of the Chinese oak silkmoth, *Antheraea pernyi* (Guérin-Méneville). Developmental time was extended, egg cannibalism increased, and fecundity decreased for diet-reared *A. chinensis*. The molecular mechanisms underlying the nutritive impact of the artificial diet on *A. chinensis* health were investigated using nutrigenomics. RNA-seq (a process of cloning, mapping, sequencing and analysis of RNA sequences) established that the biological pathways associated with differentially expressed genes (DEGs) between the pupae-fed and diet-fed treatments mapped 13,872 DEGs and annotated sequences to the reference canonical pathways in KEGG, a collection of databases dealing with genomes and biological pathways (Zou et al., 2013a). In total, 5879 sequences were assigned to 239 KEGG pathways. The pathways most represented by the DEGs were metabolic pathways (891, 15.16%) and cancer pathways (215, 3.66%) (Zou et al., 2013a).

Most upregulated DEGs in diet-fed versus prey-fed *A. chinensis* were part of 10 metabolic pathways related to vitamin metabolism. The excesses indicated by these results were remediated by reductions in the diet formulation. Another group of upregulated DEGs were part of seven pathways related to fat metabolism, including adipocytokine signaling pathway, pyruvate metabolism, fatty acid biosynthesis, glycerolipid metabolism, fat digestion and absorption, fatty acid metabolism and fatty acid elongation. These results indicate excess dietary lipid that was remediated with reductions of a chicken egg and pig liver in the diet formulation. Another group of upregulated transcripts were enriched for four pathways related to starch and sugar metabolism, including carbohydrate digestion and absorption, fructose and mannose metabolism, sugar-lipase-3, glucose transporter, and insulin and mTOR signaling. These signal excess dietary sugar and carbohydrates that were remediated with reductions of sugar in the diet formulation. Additional ingredients were also adjusted based on information extracted from the nutrigenomics data. Collectively, the formulation changes resulted in improving several parameters, including increased egg viability, and decreased egg and adult cannibalism (Zou et al., 2013a, 2019).

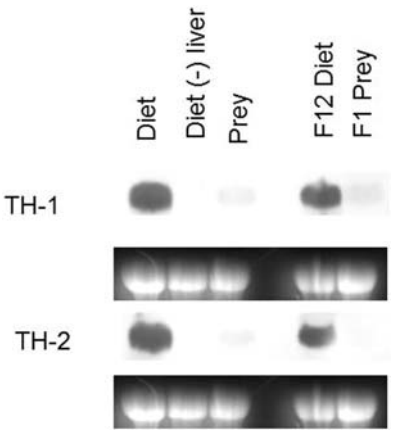

FIGURE 8.8 Expression of two *Perillus bioculatus* candidate biomarkers in response to dietary modifications. Northern blot analysis of two RNA transcripts (TH-1 and TH-2; with sequence homologies to tyrosine hydroxylase) isolated from fourth instar nymphs fed an artificial diet [Diet], artificial diet in which liver was removed [Diet (−) liver] or prey *T. ni* [Prey]. In another study, F12 generation of diet−fed fourth instar [F12 Diet] were switched to prey diet and the resulting F1 generation prey−fed fourth instar nymphs were analyzed [F1 prey]. Total RNA (7.5 μg) was separated on a 1.2% formaldehyde-agarose gel, transferred onto positively charged nylon membrane and probed with the clone indicated to the left of each panel. Equal loading was ensured by ethidium bromide stained ribosomal RNA, shown below its respective northern blot.

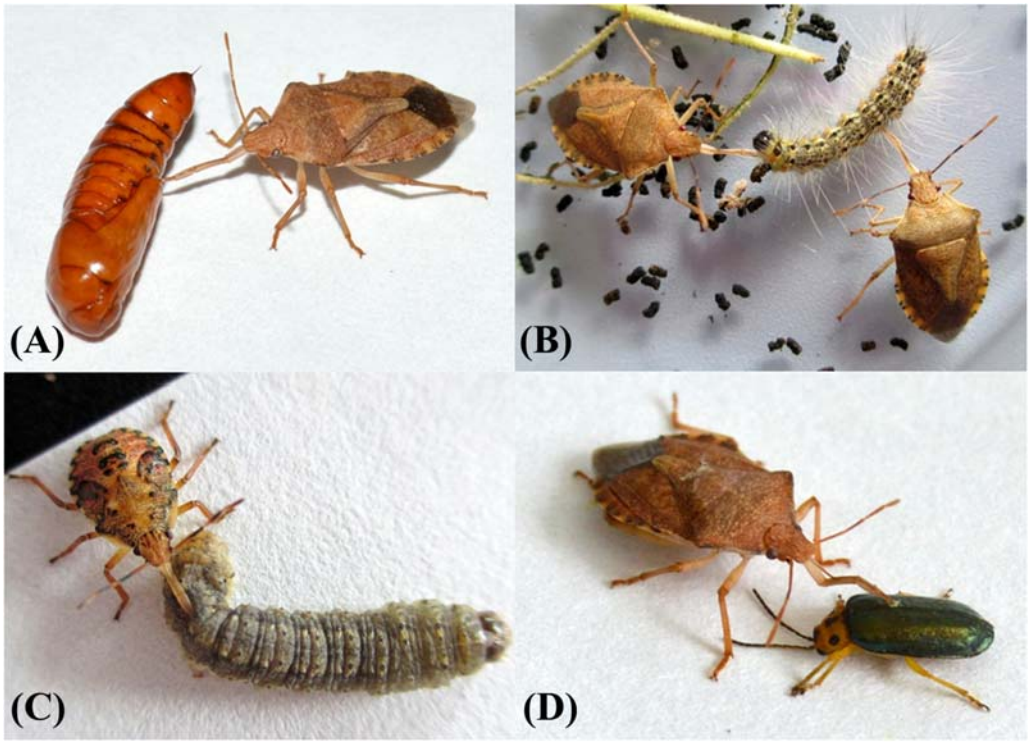

FIGURE 8.9 Predatory behavior of *Arma chinensis* (Fallou). (A) Adult is sucking pupa of *Helicoverpa armigera* (Hübner); (B) Adults are sucking larva of *Hyphantria cunea* (Drury); (C) Nymph is sucking larva of *Spodoptera exigua* (Hübner); (D) Adult is sucking adult of *Pyrrhalta aenescens* (Fairmaire).

Knowing the physiological roles of the DEGs enabled the correction of some dietary ingredients and subsequently the formulation of an improved artificial diet for *A. chinensis* (Zou et al., 2019). Although a number of potential nutrient-sensitive genes were identified, there remains the challenge that most individual genes are regulated by more than one factor or nutrient as in this case. Expression profile "signatures," defined as the characteristic suites of differential gene expression (Elliott, 2008) and the emerging field of systems biology (Kang, 2012) may help overcome this challenge.

The newly emerging field of metabolomics may compliment nutrigenomics in assisting diet formulation. The metabolome, as a snapshot of carbon flux within an organism, is the most proximal manifestation of a changing phenotype resulting from nutrition and environmental factors. We have undertaken preliminary studies (Huynh et al., in prepration) to test the application of metabolomics for refining insect diet formulations. We are examining metabolite differences between insects reared on natural and artificial diet formulations using global metabolomic analysis. Metabolite expression changes are being used to identify biochemical pathways potentially contributing to treatment differences. Candidate metabolites that are identified will be used to elucidate and characterize dietary excesses or deficiencies in a manner similar to, and in combination with, the use of results from nutrigenomics.

As more examples of biomarkers emerge one can anticipate a convergence to a set of genes and gene products that fit the criteria of good biomarkers and are indicative of specific dietary excesses or deficiencies. In many cases, these may be genes not previously thought of as predictors of nutrient quality. Currently, we are in the early stages of translating genomic, proteomic, and metabolite profiles into biomarkers that can be used to guide insect diet formulations.

8.6.3 Endosymbionts

In Chapter 3, microorganisms associated with heteropteran predators are discussed, with a focus on endosymbiotic and pathogenic interactions. Here we will address the probiotic gut microbiota as related to insect diets, although the gut microbiota represents all aspects of microbial relationships, from pathogenic to obligate mutualism. Ferreting out the complex roles of insect gut microbiota is in its initial stages and hence is not well understood, but it is commonly accepted that gut microbiota have a significant role in insect nutrition via food digestion. Hence, to assist in diet

development and optimization, some degree of gut microbiota manipulation may prove helpful. The two most common effects of artificial diet are the depletion or replacement of normal gut microbiota and the presence of foreign bacteria associated with the contamination of the diet. Cuticular microbiota may also be altered by container microhabitats, diet ingredients, and human handling. These alterations of microbiota could impact development, mating and reproduction in unpredictable ways.

Probiotic bacteria provide valuable metabolic resources for many entomophagous arthropods through a symbiotic, multitrophic interaction between the insect and its food (Douglas, 2018). Although awareness of gastric caeca containing bacteria in Heteroptera dates back nearly 100 years (Glasgow, 1914) for the predatory Heteroptera details of the relationship between the insect and its gut microbiota remain undefined. Genomic and proteomic methods (discussed above) have recently been used to accumulate information on the gut microbiome and have resulted in the characterization of indigenous and transient populations of bacterial communities in the insect alimentary canal, the detection of new species, and more frequent links to biological roles.

Gut bacterial communities adapt by the transfer of plasmids and other content between bacterial strains (Dillon and Dillon, 2004). We reason this relationship and adaptation between the insect and its microbiota is critical to the insectary production of insects optimized for fitness. However, using microbiota to optimize the production of insects will require a better understanding of the molecular relationships between the insect and its microbiome. The complexity and plasticity of the gut microbiota and the influence that rearing has on gut microbiota will challenge such studies.

It is widely assumed that insect gut microbiota are derived from the surrounding environment, including food, but also microbials passed from one generation to the next; usually associated with the egg. Most of what we know today about heteropteran gut endosymbionts is from studies with phytophagous pentatomids (Prado and Almeida, 2009), although preliminary studies have begun to document the effects of rearing on gut endosymbionts in the heteropteran predator *P. maculiventris* (Coudron, unpublished). There is much to learn before we will be able to effectively use endosymbionts to optimize the production of entomophagous arthropods. For example, in addition to the identification of microbial species, the following would be important to know for production purposes: degree of persistence of ingested strains; particular niches in the gut where specific strains colonize; which microbial species colonize the gut habitat; interactions among bacteria, the role of indigenous gut microbiota in preventing or suppressing infections, the influence of food and the effect of antibiotics in artificial diet formulations on the gut microbiome.

8.7 Concluding remarks

In this chapter, we have presented concepts of biochemistry, analytical chemistry, biophysics, mathematics, biology, physiology, demographics, behavior, genetics, genomics, dietetics, and more, all applied to the development and improvement of artificial diets for entomophagous arthropods. All the methods discussed in this chapter focus on advancing artificial diets from rearing to mass production. Advancing the technology of artificial diets to the commercial application requires skills in engineering, artificial intelligence, robotics, food processing, and materials science. It is evident that advancing the development of artificial diets is a multidisciplinary enterprise, requiring the integration of multiple skills and the collaboration of teams to fulfill the goals of researchers and industry for the use of beneficial arthropods. The authors of this chapter wish to convey the importance of incorporating new ideas and technology in our efforts to meet those goals. The accelerated pace at which the field of artificial diets for arthropods is advancing makes us optimistic that the inherently complex challenge of diet development will be met in the next decade and subsequently those goals achieved.

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Chapter 9

Concepts and methods of quality assurance for mass-reared parasitoids and predators

Norman C. Leppla
Entomology and Nematology Department, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, FL, United States

9.1 Introduction

Quality assurance for mass-reared arthropods expands concepts previously incorporated into a total quality control system for insect production (Leppla and Fisher, 1989; Leppla, 2003, 2009) and increases emphasis on postproduction use of parasitoids and predators in integrated pest management (Fig. 9.1). Quality assurance includes all of the factors that comprise product development, production, delivery, application, and evaluation, as well as feedback on their effectiveness (Feigenbaum, 1983; Webb, 1984; Juran and Godfrey, 1998). These factors can be measured and managed to assure the performance of mass-produced arthropods and to detect changes that could compromise their effectiveness in the field. Parasitoids and predators must be healthy enough to survive for a specified period after being released and effective in locating and killing or significantly injuring prey or hosts, and possibly reproducing. They are required to withstand handling, packaging, storage, shipment over long distances, transport to the field, and mechanical application (Messing et al., 1993; van Lenteren and Tommasini, 2003; Rull et al., 2012). Their postproduction quality can therefore be determined by characteristics, such as a number of live organisms per package and their age, weight, size, tolerance to a wide range of temperature and humidity, resistance to environmental hazards, dispersal (Zboralski et al., 2016), longevity, fecundity, and impact on target pests (Parra and Coelho, 2019). However, quality assurance in the mass production of beneficial arthropods is not limited to measurements and tests of organisms, rather it is the design and management of systems that produce and deliver products that satisfy customer expectations (Deming, 1986; Burt, 2002).

Quality assurance for mass-produced arthropods was created to improve the reliability of producing and using products that meet required specifications and standards. High-quality arthropods were not always available when their widespread use in pest management was a new concept (Smith, 1966; Knipling, 1966, 1979; King and Leppla, 1984; Singh and Moore, 1985; Leppla and De Clercq, 2019). Reliable arthropod production was achieved only after industrial quality control procedures designed to consistently produce nonliving products were adapted for mass rearing insects (Boller and Chambers, 1977a; Boller and Chambers, 1977b; Chambers, 1975, 1977; ANSI/ASQC, 1987), keeping in mind the physiology, ethology, and ecology of the production and target populations. From the beginning to the present, a primary goal has been to minimize the genetic divergence of the founder and laboratory populations, particularly if behavioral traits are to be preserved (Boller, 1972; Ashley et al., 1973; Mackauer, 1976; Huettel, 1976; Leppla and Ashley, 1989; Castañé et al., 2014). Much of the evolution of quality assurance for mass-produced arthropods can be obtained from conference and workshop proceedings of the International Organization for Biological Control (IOBC) Global Working Group on Mass Rearing and Quality Assurance (MRQA) (1981 to present, most available at https://www.iobc-global.org/global_wg_mrqa.html). Boller and Leppla (2006) traced the history of the MRQA, formerly the Working Group on Quality Control of Mass-Reared Arthropods and Arthropod Mass Rearing Quality Control Working Group (AMRQC).

Mass rearing was defined by Chambers (1977) as the "production of insects competent to achieve program goals with an acceptable cost/benefit ratio and in numbers per generation exceeding ten thousand to one million times the mean productivity of the native population female." Moreover, mass production, in contrast to small-scale rearing

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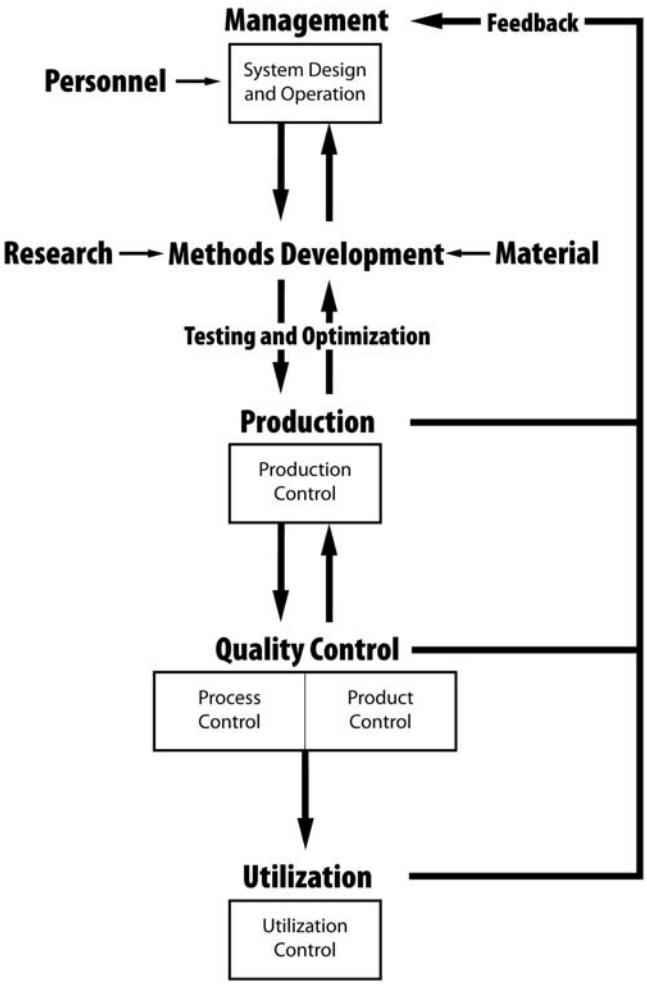

FIGURE 9.1 A quality assurance system for mass-reared arthropods expanded from total quality control for insect production (Leppla, 2009), with increased emphasis on utilization, that is, customer support and feedback. Management provides administrative support for designing and operating the entire system. The personnel division reports directly to management. The research and material divisions provide input to methods development which is responsible for testing and optimizing rearing facilities, equipment, materials and methods. The production division performs standard operating procedures for mass rearing the arthropods and controls required production inputs and processes. Rearing processes and products are monitored by the quality control division to assure that the arthropods meet established specifications and standards. The utilization division assesses product quality along the supply chain and assures that customer expectations are met. Management receives feedback on product quality from the production, quality control and utilization divisions enabling decisions to be made about adjusting the system.

accomplished by one person or a small group, is an industrial activity, usually involving a single species to support biologically based pest management, in factory-like facilities with controlled environments, artificial diets and oviposition substrates, mechanized equipment, and a succession of essential processes performed by separate work units (Nakamori et al., 1992; Lacasse et al., 2006; Leppla, 2009). Mackauer (1972) defined mass production of entomophagous insects as the rearing per generation cycle of one million times the mean number of offspring per female. Although this chapter describes quality assurance for mass-produced arthropods, the principles can be applied to any magnitude of rearing, especially the production of parasitoids, predators and herbivorous natural enemies.

The nucleus of a quality assurance program for mass-reared arthropods is the production capability and associated production, process and product quality control (Leppla and Fisher, 1989; Bruzzone et al., 1993). A few species of arthropods have been mass-reared in "biofactories" that employ several hundred workers during three shifts per 24-hour day, seven days per week (Leppla and Ashley, 1989; IAEA, 2008). The processes performed in these biofactories are separated in time and space, so mass-rearing systems are required to be highly coordinated. Efficient production requires that the arthropods have high levels of fertility and fecundity, exhibit rapid and synchronous development, are easy to harvest, and sustain high yields (Smith and Nordlund, 2000; La-Spina et al., 2018). Less industrialized rearing and associated quality control programs have been used successfully to produce beneficial arthropods, such as *Trichogramma* spp. (Morrison, 1985; Laing and Bigler, 1991; Qiu et al., 1992; Pavlik, 1993; Wuehrer and Hassan, 1993; Bigler, 1994; Cerutti and Bigler, 1995; Bourchier et al., 2000; Liu and Smith, 2000; Prezotti et al., 2002; Kolliker-Ott et al., 2004). However, the amount of insect mass-produced is greatest for the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and screwworm fly, *Cochliomyia hominivorax* (Coquerel), used for sterile male release. Production of the Mediterranean fruit fly has relied on a well-developed quality control program to reach several billion per week (Rendon et al., 2005). Quality control programs also have been adopted widely for mass-

producing the codling moth, *Cydia pomonella* (Linnaeus), (Hathaway et al., 1973; Bloem et al., 1998), mosquitoes (Dame, 1989; Carvalho et al., 2014), tropical fruit flies (Leppla, 1989; Caceres et al., 2007) and many other arthropod species (Moore et al., 1985; Zlotin and Chepurnaya, 1995).

The purpose of this chapter is to describe the principles of quality assurance for mass-produced arthropods, especially as they apply to produce parasitoids and predators. Quality assurance encompasses every aspect of arthropod mass production and utilization for augmentation biological control. Its application can accelerate the use of commercial natural enemies (Penn et al., 1998; van Lenteren, 2012) and enable increased growth of the industry (Hoy et al., 1991). Markets for mass-produced natural enemies are increasing but potential customers are seeking assurance that the products are reliable and effective (van Lenteren et al., 2018). For this chapter, customers are consumers or clientele who do not necessarily pay for the products, for example, government biological control programs as well as paying customers. Regardless, it is essential for customers to be involved in assuring the quality of products they obtain because products must meet required specifications and standards. Customer satisfaction ultimately depends on how well the beneficial arthropods suppress arthropod and weed pests in agriculture, communities, and environmental areas, such as wetlands and rangelands. This chapter provides a general framework for building a complete quality assurance system, including examples of product quality control for natural enemies and associated quality assessment and control data acquisition and analysis. Quality assurance systems can be reviewed periodically according to a basic guide that is included. The chapter concludes with a description of research priorities for mass-reared natural enemies. Excluded are alternative rearing methods and studies of parasitoid and predator field effectiveness.

9.2 Quality assurance in the marketplace

As in any business, including commercial biological control, successful products and services are defined by their usefulness and profitability (Penn et al., 1998). Natural enemy product effectiveness, however, often is difficult to quantify because of widely varying pest damage, naturally occurring biological control, natural enemy dispersal, and many other variables. Consequently, standards have been developed to specify the quality of commercial natural enemies produced and delivered to the customers. The Association of Natural Biocontrol Producers (ANBP) developed standards for selected commercial natural enemies using a system developed by ASTM International (http://www.astm.org). Moreover, at least one company, BioBee Biological Systems (http://biobee.com), uses International Organization for Standardization, ISO 9001 (http://www.iso.org/iso-9001-quality-management.html) and IOBC international standards for mass-producing natural enemies (van Lenteren, 2003b) (http://users.ugent.be/~padclerc/AMRQC/images/guidelines.pdf). ISO International defines a standard as "a document that provides requirements, specifications, guidelines or characteristics that can be used consistently to ensure that materials, products, processes and services are fit for their purpose." Specifications are essential characteristics and tolerances, limiting values and other definitions for materials, products, services, processes, systems or persons, contained within the provisions of a standard (Juran et al. 1979).

Quality assurance systems for commercial natural enemies include all aspects of production, including completion of standard operating procedures; the results of monitoring rearing processes; and attaining the performance standards for resulting products. Also included are contractual agreements, such as on-time delivery of the required number of living parasitoids or predators. Typical production control characteristics are ranges of temperature and relative humidity, and for process control the yield per rearing container. For product control, standards usually describe the following measurable characteristics: species or strain identity and purity, age, size, weight, motility, survival, host location, and level of parasitism (Smith, 1996). These kinds of characteristics can be used to evaluate products and their sources, and compare products over time to detect changes. Requirements for postproduction quality assurance of mass-produced arthropods include suitable packaging, careful handling, rapid shipment, reliable delivery, product care, appropriate application, and effectiveness (Brazzel et al., 1986; Bloem et al., 2002; Anonymous, 2003; FAO/IAEA/USDA, 2003; Enkerlin and Quinlan, 2004; Enkerlin, 2007; Blomefield et al., 2011). Cost and payment terms also affect customer satisfaction. For *Encarsia formosa* Gahan, cost per functional insect was proposed as a useful quality guideline (Vasquez et al., 2004).

The acceptance of natural enemy products in the marketplace results not only from meeting written standards but also from successful use of these products by satisfied customers (Penn et al., 1998). There were at least 30 large (>10 employees) biological control companies in the world that produced and sold about 200 arthropod species of natural enemies (van Lenteren, 2012) but this has increased to 10 companies with more than 50 employees and 350 products in the marketplace (van Lenteren et al., 2018). The largest producer employs about 1400 people. Currently, global sales are conservatively estimated to be at least $800 million/year, based on an anticipated 15%−20% annual growth rate from $50 million in 2001 (van Lenteren, 2003a). Mass-produced parasitoids and predators are sold to farmers, ranchers,

nursery operators, grower associations, crop advisors, pest managers, homeowners, and others for use in field and glasshouse crops, orchards, outdoor landscapes, interior plantscapes, pastures, livestock operations, forests, waterways, horse farms, and a few other appropriate situations. Accordingly, the acceptable quality of commercial parasitoids and predators is determined by these markets. Natural enemies will continue to be mass-produced and used in greater quantities if they are affordable and suppress pests to levels below acceptable thresholds (van Lenteren, 2012).

9.3 Customer involvement in quality assurance

The quality of mass-produced parasitoids and predators is not only intrinsic but also related to the needs and expectations of the customers. Therefore, to maintain sales, producers and suppliers are required to not only provide high quality products but also make sure that the customers are satisfied (Penn et al., 1998; Bolckmans, 1999). Customers are more likely to be satisfied if they are involved and educated in the evaluation and application of the natural enemy products they purchase (Bolckmans, 1999). Based on experience, the most helpful suppliers develop application guidelines and provide the required number of natural enemies to match the extent of a pest infestation. Especially critical and easy to measure are the numbers of living and dead natural enemies in a package. Every package should be sub-sampled prior to shipment to ensure that it contains at least the number ordered; the normal target is about 15% overpacking. During shipment, natural enemies are exposed to darkness, an abnormally high density, reduced oxygen concentration, potential temperature and humidity extremes, and possibly to condensation. Natural enemies, therefore, are removed from the shipping container immediately when received and assessed by the customer for viability. Customers can use quick and simple assessment methods that enable them to evaluate the products and expeditiously return feedback about product quality to the suppliers who in turn inform the producers (van Schelt and Mulder 2000; Buitenhuis, 2014). For some products, such as predatory mites or beetles, this is a short term evaluation with a motion indicating survival and some level of fitness. A longer period may be required to confirm the emergence and movement of parasitic wasps and midges. To facilitate these kinds of evaluations and maximize the effectiveness of natural enemy products, customers need better information and training (Bolckmans, 2003).

Customer feedback is an essential driver of product improvement. For example, a vegetable grower organization in British Columbia, Canada provided feedback to four natural enemy producers on the quality of *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) and *E. formosa* (Hymenoptera: Aphelinidae) supplied for use in their glasshouses (Glenister et al., 2003). The producers were identified only by code and the grower's quality consultant notified them within a few weeks of the quantity of *E. formosa* in their shipments. During a 3-year trial, from 1997 to 1999, the quantity of *E. formosa* from one company improved from less than 5% of the number ordered to consistently 100%. Products from the second company followed the same pattern, achieving 100% in 1999. In 1997, only about 50% of the *E. formosa* shipments from the third company contained the expected number but more than 80% was achieved in 1998 and 1999. For the fourth company, the average number of *E. formosa* actually declined from about 50% the first year to below 20% by the end of the study. Emergence also was measured and exhibited the same general pattern as quantity, that is, packages with low numbers of insects had low levels of emergence. Unfortunately, the quality consultant was not able to measure the relative fecundity of the *E. formosa*. The performance of these companies probably was inconsistent during the study because there were changes in personnel, rearing capabilities, host sources or quality, weather, packaging, delivery routing, and quality assurance programs. In response to rapid feedback, it can be possible for producers to control the variables that determine product quality and reliably ship specified numbers of high-quality natural enemies. Ideally, the most successful producers and suppliers provide technical support to help growers assure that the natural enemies they purchase are effective and take rapid corrective action if necessary.

9.4 Building a complete quality assurance system

Quality assurance in the mass production of arthropods, with minimal emphasis on product utilization, has been referred to as "total quality control in insect mass production" (Leppla and Fisher, 1989; Leppla, 1989, 2003). This organizational structure was adapted from manufacturing industries and similarly involves designing and operating systems that satisfy customer requirements for product performance (Fig. 9.1). The production capability is developed and optimized by continuously improving environmental conditions, testing new methods and materials, monitoring standard operating procedures, evaluating the resulting products, determining product effectiveness, and providing feedback to make operational adjustments (Neuenschwander et al., 1989; Gonzalez-Zamora and Castillo, 2018). In the spirit of W. E. Deming, J. M. Juran, A. V. Feiningbaum and other pioneers of industrial quality assurance, every detail of an arthropod mass production and utilization system is designed and managed to maximize product quality and effectiveness (Feigenbaum,

1983). Juran et al. (1979) defined quality assurance as "the activity of providing, to all concerned, the evidence needed to establish confidence that the quality function is being adequately performed." Accordingly, the quality function for arthropod mass production and utilization encompasses all of the following divisions of the system, regardless of how simple or complex (Leppla and Fisher, 1989) (Fig. 9.2).

9.4.1 Management

The first step in establishing an arthropod quality assurance system is to reach a written agreement on exactly what customers need and expect in terms of product specifications and quality standards (Anonymous, 2006), product delivery and evaluation, customer support remotely and in the field, and responsiveness to needed adjustments (Leppla, 2003). Management must fully participate in planning the organization, establishing policies for its operation, and making necessary changes based on feedback from the production, quality control and utilization divisions. Standard operating procedures for production are written cooperatively by the facility managers and key employees, including a sequence of detailed rearing processes, associated assignments, and schedules in the form of instructions and check sheets (Minno and Holler, 1987; Fisher, 2009b). Schedules are arranged to provide coordination between processes accomplished at different times or locations. Considerable effort is devoted to defining competencies for rearing quality insects and selecting, training, motivating, evaluating, and compensating appropriate personnel (Fisher, 1984a; Singh and Ashby, 1985). This kind of organization and planning extends from management through production and all of the postproduction activities. Management provides ongoing administrative support for the entire quality assurance system.

Management can analyze the market and establish a quality assurance system by developing a comprehensive business plan. An example of producing sterile insects is the "Model Business Plan for a Sterile Insect Production Facility" (IAEA, 2008). It describes commercial issues, such as ownership, costs and financing, organizational structure, site selection, intellectual property, liability insurance, and markets and pricing for the primary and by-products. Additional considerations for arthropod mass production include accessibility of supplies, facility design and operation (Fisher, 1984a), environmental requirements, health hazards (Reinecke, 2009; Suarthana et al., 2012), labor availability, employment conditions, and economics (Nasreen et al., 2011). The business plan includes a clear description of how the system will be managed and provided with adequate resources (Singh and Ashby, 1985). It will define quality assurance for

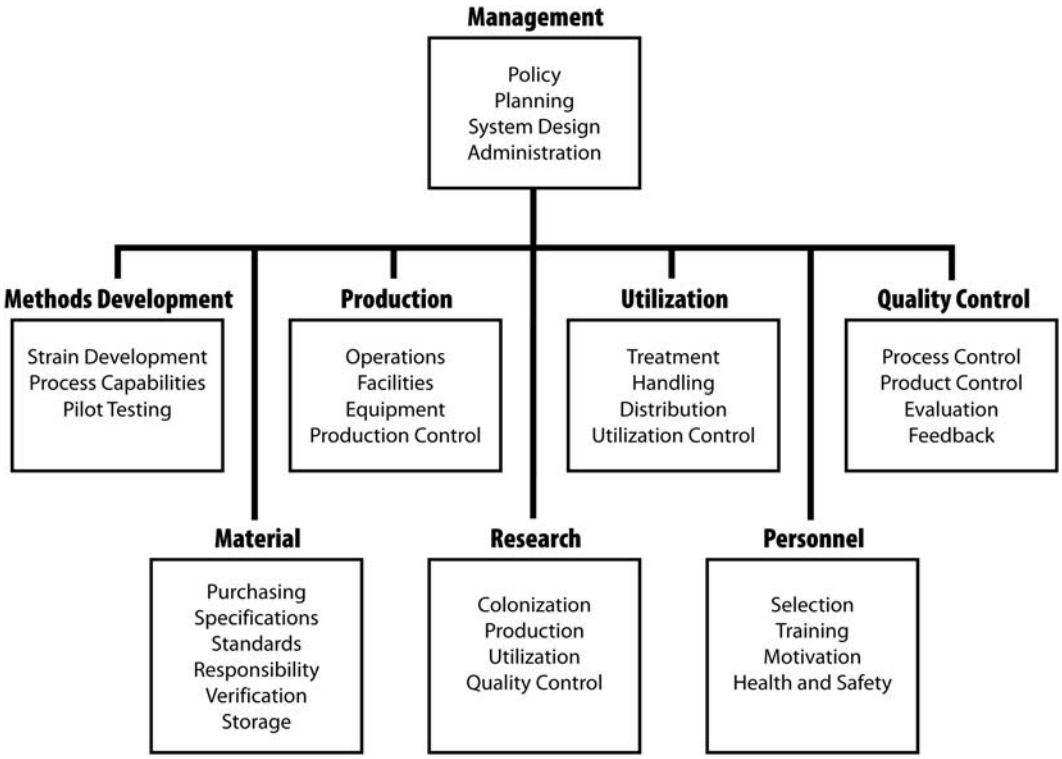

FIGURE 9.2 Divisions and associated functions of a quality assurance system for arthropod mass production described initially in total quality control for insect production (Leppla and Fisher, 1989).

producing and delivering high-quality products, improving product effectiveness, and increasing associated customer satisfaction (Penn et al., 1998).

9.4.2 Methods development

An arthropod mass rearing program typically has a distinct methods development division that conducts process capability studies to establish, maintain, and evaluate prospective colonies, as well as improve associated facilities, materials, equipment, and procedures (Goodenough, 1984; Griffin, 1984b; Harrell and Gantt, 1984; Edwards et al., 1996). Founder populations collected at several sites by the research division are propagated, the best being selected for mass rearing or to replace the colony in production (González-Cabrera et al., 2017; Gols et al., 2019; Ebrahimi et al., 2019). Methods development also can support utilization by helping to invent new ways of treating (Suárez et al., 2019a), handling, packaging, distributing, and evaluating the natural enemy products. Potential advancements for arthropod production, delivery and evaluation, including those from research, are pilot tested within the existing quality assurance system before being adopted. Unlike the quality control division, methods development does not have a "troubleshooting" responsibility to correct immediate shortcomings in implementing established protocols. On the contrary, it seeks to progressively increase product quality and reduce costs by optimizing the system (Gonzalez-Zamora and Castillo, 2018). Since suitable facilities are essential and expensive, methods development gives special consideration to their design, construction, maintenance, and, if necessary, modification (Griffin, 1984a; Owens, 1984; Goodenough and Parnell, 1985; Fisher and Leppla, 1985; Anonymous, 2004; Fisher, 2009a).

9.4.3 Material

Materials and supplies are selected, purchased, received, inventoried, stored, and used according to the quality assurance system, for example, anticipated production levels, reliability of suppliers, distance from sources, required lead times, etc. Specifications and standards for materials and supplies are described in detail within written purchasing contracts. Suppliers are responsible for the quality of their products and are not allowed to unilaterally decide to substitute alternatives. Every shipment is labeled with received and "use by" dates. Rather than being assumed, required quality specifications and standards for all materials are verified on receipt, by bioassay if necessary. Even paper products and water can be contaminated with substances, such as pesticides and other toxicants that can reduce the quality of mass-reared arthropods. It is particularly difficult to control the quality and purity of dietary ingredients (Brewer and Lindig, 1984; ODell et al., 1997; Cohen, 2004; Inglis and Sikorowski, 2009). Detailed records are kept on all materials and supplies used in arthropod production and utilization (ODell, 1992).

9.4.4 Production

Mass production of beneficial arthropods must be reliable and result in high-quality products (Knipling, 1984; Leppla, 2003; Hagler, 2009; Vasquez and Morse, 2012). To increase reliability and reduce variability, standard operating procedures are performed diligently and deviations corrected rapidly (Anonymous, 2006). Production control check sheets are particularly useful for identifying the source of a problem because most production deficiencies are caused by failure to perform standard operating procedures. However, when production problems occur beyond environmental or equipment failures and procedural mistakes, management requests diagnostic assistance from other divisions, for example, quality control, methods development and research, to quickly identify the causes. Production facilities are designed to be durable, efficient and easy to clean, and every environment is kept within the specified range of temperature, RH, photoperiod, and airflow and purity (Fisher, 2009a). Rooms with different rearing processes should have separate air handling equipment and be arranged in a linear sequence from ultra-clean to less clean with traffic patterns that do not allow employees to backtrack. Ultra-clean areas require high-efficiency particulate absorbing (HEPA) air filtration (Goodenough and Parnell, 1985) or laboratory benches with HEPA-filtered laminar airflow. Linear workflow traffic patterns and sanitation are essential for arthropod natural enemy production (van Frankenhuyzen et al., 2004; Inglis and Sikorowski, 2009), especially tritrophic systems that incorporate plants and phytophagous hosts (Li, et al., 2020).

9.4.5 Research

Applied research that supports arthropod mass production and quality assurance is conducted to improve colonization, production, utilization and quality control. More effective natural enemy species or strains are sought, along with better

colonization procedures (Hoffman et al., 1984; Leppla, 1989; Nunney, 2002; Benedict et al., 2009). The goals of research in support of production generally are to adapt materials, invent the equipment and develop methods that improve the system and cost less (Vacari et al., 2012). Typical examples are plants or diets that produce more natural enemies or sometimes suitable alternative hosts (Bernal et al., 1999; Blossey and Hunt, 1999; Karamaouna and Copland, 2000; Serrano and Lapointe, 2002; Wheeler, 2003, 2006; Geden and Kaufman, 2007; Giang and Ueno, 2007; Van Hezewijk et al., 2008; Grenier, 2009; Henry et al., 2010; Wyckhuys et al., 2011; Costa et al., 2016; Cai et al., 2018). Production, process and product control procedures can be improved most effectively by conducting research within mass-rearing facilities (Couillien and Gregoire, 1994; Miyatake, 2011). Without supporting research, improvements in the production and use of mass-reared arthropods will be incremental and based on the methods development division and customer feedback. The research division may have overlapping responsibilities with the methods development division, or they may be combined depending on the quality assurance system.

9.4.6 Utilization

Mass-produced arthropods must be competent to achieve program goals (Chambers, 1977), as prescribed by established specifications and standards. Quality assurance is more than determining if product requirements are met initially; it also involves the maintenance of postproduction quality (Dominiak et al., 2011). Uncomplicated and rapid product control evaluations indicative of natural enemy performance are used to assess product quality prior to shipment and at key points along the supply chain to application in cropping systems (Bourchier et al., 1993; Dutton et al., 1996; Bennett and Hoffmann, 1998; Mansfield and Mills, 2002). Product quality can be affected by treatments, such as feeding adults, cold storage, and irradiation (Cancino et al., 2009, 2020; Suárez et al., 2019a; Suárez et al., 2019b; Sakaki et al., 2019). The performance of parasitoids can be improved by exposing them to the host or host environment prior to release (Tanga et al., 2013; Benelli et al., 2014; Pan et al., 2014; Giunti et al., 2016; Masry et al., 2019). Automated counting, handling and packaging equipment are required to efficiently collect and ship large numbers of arthropods. Additionally, for natural enemies, it often is necessary to differentiate predators from prey and parasitized from nonparasitized hosts (Yazdani et al., 2015). Differentiation of males from females may be required for parasitoids because host quality can affect sex ratios (Poncio et al., 2018; Heimpel and Lundgren, 2000; Lundgren and Heimpel, 2003). Natural enemy products should at least be checked for identity, purity and quantity before being shipped. During transport, containers must both preserve product quality and match the requirements of the shipper. Due to their short time of effectiveness, natural enemies usually are shipped via air carriers. Effectiveness of parasitoids and predators can be assured further by optimizing the number applied and the rate and timing of applications, using mechanized application equipment or other methods that ensure complete coverage, and monitoring natural enemy/pest ratios.

9.4.7 Personnel

Quality assurance for arthropod mass production and utilization depends on selecting, training, and motivating appropriate personnel, and protecting their health and safety (Fisher, 2009b; Reinecke, 2009). Arthropod mass rearing is highly repetitive and often tedious, requiring attention to detail and consistent accuracy. It is somewhat analogous to other monotonous industrial farming processes, particularly those involving living organisms, such as potting plants or tending confined animals. Therefore, employees who do not easily tolerate repetitive tasks are unlikely to be successful in the material, production, personnel, and quality control divisions but may fit in management, methods development, research, and utilization. A potential employee is asked questions designed to determine personality fit (Fisher, 2009b). Some employees enjoy routine tasks, such as those involved in rearing arthropods and work well together in small groups. In these cases, it can be extremely disruptive to add an employee who does not fit. Regardless of personality type, however, employees are to be competent and diligent in performing their assigned tasks (Cohen, 2001). Exposure to respiratory irritants, such as moth scales and other health risks, are to be minimized through protocols and the use of protective equipment (Wolf, 1984, 1985; Kfir, 1994; Suarthana et al., 2012).

Arthropod mass rearing has become a profession (Cohen, 2001), especially to support areawide pest management (Robinson and Hendrichs, 2007; Leppla et al., 2009). Competence is gained mostly "on-the-job" by working with experienced employees, although training can be accelerated considerably by observing other quality assurance systems, studying technical publications, networking with arthropod rearing specialists, and participating in formal classes, for example, the Insect Rearing Workshop at Mississippi State University, Mass Rearing of Insects Workshop at Stellenbosch University, and courses at institutions of higher education, for example, the Insect Rearing Education and Research Program, Department of Entomology and Plant Pathology, North Carolina State University (Cohen, 2018). A

matrix of competencies can be developed with the subject matter of training in the left column and a hierarchy of positions across the top row (Juran et al., 1979). Applied to arthropod mass production, the competencies column would include: adaptability, customer focus, independence, initiative, problem-solving, quality of work, teamwork, and understanding of rearing principles (Fisher, 1984b,2009b). The competencies for each position can be described in the internal squares of the matrix. The highest level position, manager, should master the entire production system, including facility design and management, nutrition and diets, microbial management, quality control, applied population genetics, environmental biology, and health and safety (Schneider, 2009). It has been suggested that certification for each position could be earned by mastering all of the competencies at the corresponding level in the matrix. This kind of training and certification could provide motivation and justify increased advancement and compensation for insect rearing specialists (Cohen, 2001).

9.4.8 Quality control

The goal of quality control is to maximize yields of high-quality arthropods by rapidly detecting and correcting deviations from standard operating procedures and minimizing variability in production (Bigler, 1989; Sagarra et al., 2000; Bueno et al., 2006). It is accomplished by monitoring production materials and procedures, periodically sampling arthropod stages during development to ensure process control, and evaluating living organisms or associated end products through product control (Leppla, 2009). Monitoring based on efficient and accurate sampling and evaluation throughout production is required to maintain key characteristics of natural enemies within specified ranges (Pashley and Proverbs, 1981). The frequency and extent of quality control evaluations depend on the stability of the system. Sampling, analysis, record keeping, and reporting should be efficient and limited to the amount of information needed to maintain product effectiveness. The quality control division is responsible for rapid data acquisition, analysis and feedback in appropriate formats. Extending beyond quality control, quality assurance incorporates all actions that affect the effectiveness and customer acceptance of the product (Table 9.1).

9.5 Quality assessments of mass-reared natural enemies

At least minimal product quality control guidelines have been developed for many commercially produced natural enemies (Bigler, 1992; Buitenhuis, 2014; van Lenteren et al., 1993; van Lenteren, 2003b; Leppla and Larson, 2004) (Table 9.2); however, they must be improved and adapted for use in specific situations. The guidelines specify a limited number of parameters that can be measured to indicate consistent quality for a species. Life history and morphological characteristics are easier to measure than behavior (Noldus, 1989; Lux, 1991) and can be good indicators of quality, for example, number of adults, rate of emergence, the proportion of live insects, size or weight, sex ratio, and longevity. Highly variable characteristics that are relatively difficult to measure, such as fertility and fecundity, are used only if necessary to monitor production and if the natural enemies are expected to reproduce in the field. The life history and behavior of mass-produced natural enemies will be consistent in reasonably stable production systems (Gandolfi et al., 2003), yielding quality control data that can be used to detect deviations from historical values.

The quality of mass-reared parasitoids and predators has been assessed partially by conducting postshipment product quality control evaluations. In an unprecedented 2.5-year study, three unidentified surrogate customers ordered the following species from three or four different companies: *E. formosa*, *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), and *Hippodamia convergens* Guerin (Coleoptera: Coccinellidae) (O'Neil et al., 1998). The number, identity and stage of insects received were evaluated and, depending on species, assessments were made of emergence, sex ratio, survival, reproduction and hyperparasitism. Initially, few adult parasitoids had emerged during shipment, regardless of species. For *E. formosa*, the number of pupae received was equal to the number ordered or considerably greater, emergence averaged 41.3% and survival of adults after two days was 89%−95%. Shipments of *T. pretiosum* periodically contained fewer insects than were ordered and the average survival was 86%. Considering shipments of *H. convergens*, 50%−81% were alive when received and 82%−96% survived for two days, although 9%−22% were parasitized. All of the presumptive *C. carnea* adults received from the three companies actually were *C. rufilabis* (Burmeister). Since there often was considerable variability in the products, the study concluded that "working relations between producers, evaluators, and customers would benefit all." Further, "Defining realistic expectations for the quality of commercially available natural enemies will serve to expand their use in augmentative biological control and integrated pest management programs."

E. formosa was included in a subsequent quality assessment of whitefly parasitoids, along with *Aphidius colemani* Viereck (Hymenoptera: Braconidae) and *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) (Vasquez et al.,

TABLE 9.1 Quality assurance program for natural enemy producers and suppliers.

Quality assurance actions	Frequency of actions		
Production and process control	**Routinely**	**Periodically**	**Possibly**
Have up-to-date standard operating procedures	X		
Use check sheets to monitor rearing processes[a]	X		
Record daily production data	X		
Track changes in production[b]	X		
Review production with staff	X		
Improve product quality based on staff feedback		X	
Product Control			
Conduct product quality control assessments	X		
Have established minimum thresholds of quality	X		
Use standard shipping and handling procedures	X		
Customer Service			
Add customer feedback forms to shipments	X		
Add product use instructions to shipments	X		
Have an established customer service program[c]	X		
Improve product quality based on customer feedback		X	
Research			
Maintain internal methods improvement[d]		X	
Conduct publishable research			X
Communicate research needs to outside researchers		X	
Collaborate with researchers without funding projects		X	
Contract for research and provide funding		X	
Outreach			
Provide materials for training activities[e]		X	
Provide products for training activities		X	
Participate in training activities[f]		X	
Describe quality assurance program		X	

[a] *Rearing production and processes check sheets include materials, environments, equipment and procedures.*
[b] *Tracking by quality control charts, basic statistics or at least graphs.*
[c] *Customer service program with data on the problems and solutions.*
[d] *Internal staff members recommend improvements to management.*
[e] *Training materials typically include product descriptions, target pests and application procedures.*
[f] *Training activities include trade shows, field days, demonstrations, classes, and other educational venues.*

2004). Tests from the IOBC international standards were used to assess product quantity, including total adult emergence and sex ratio (van Lenteren, 2003b; van Lenteren et al., 2003), in addition to recording the number of insects that flew. The length of the tibia on a hind leg of *E. formosa* was measured as an indicator of body size. The arrival of the *E. formosa*-parasitized whitefly pupae at the expected time was highly variable, depending on the company, and only three of the six companies provided detailed information on how to handle and use their products. Parameters that varied significantly between companies were adult emergence on arrival, parasitized whitefly pupae in the container, and insects that could fly; however, total emergence, sex ratio and tibia length were not significantly different. Some shipments of *E. formosa* contained *Eretmocerus* spp., also a whitefly parasitoid. Of the 1500−5000 *E. formosa* ordered,

TABLE 9.2 Selected natural enemies that have been produced commercially and consequently have at least minimal quality standards.

Natural enemy species	Order: family
Amblydromlus limonicus (Garman & McGregor)	Acarina: Phytoseiidae
Amblyseius andersoni (Chant)	Acarina: Phytoseiidae
Amblyseius(Neoseiulus) californicus (McGregor)	Acarina: Phytoseiidae
Amblyseius (Neoseiulus) cucumeris (Oudemans)	Acarina: Phytoseiidae
Amblyseius (*Neoseiulus*) *degenerans* Berlese	Acarina: Phytoseiidae
Amblyseius (Neoseiulus) fallacis (Garman)	Acarina: Phytoseiidae
Amblyseius swirskii Athias-Henriot	Acarina: Phytoseiidae
Anagyrus pseudococci (vladimiri) Triapitsyn	Hymenoptera: Encyritidae
Anthocoris nemoralis (Fabricius)	Hemiptera: Anthocoridae
Aphelinus abdominalis Dalman	Hymenoptera: Aphelinidae
Aphelinus milinus DeBach	Hymenoptera: Aphelinidae
Aphidius colemani Viereck	Hymenoptera: Braconidae
Aphidius ervi (Haliday)	Hymenoptera: Braconidae
Aphidius matricariae Haliday	Hymenoptera: Braconidae
Aphidoletes aphidimyza (Rondani)	Diptera: Cecidomyiidae
Aphytis lingnanensis Compere	Hymenoptera: Aphelinidae
Aphytis melinus DeBach	Hymenoptera: Aphelinidae
Carcinops pumilio (Erichson)	Coleoptera: Histeridae
Chrysoperla carnea Steph.	Neuroptera: Chrysopidae
Chrysoperla rufilabris (Burmeister)	Neuroptera: Chrysopidae
Cotesia plutellae (Kurdjumov)	Hymenoptera: Braconidae
Cryptolaemus montrouzieri Mulsant	Coleoptera: Coccinellidae
Cybocephalus nipponicus Endrody-Younga	Coleoptera: Cybocephalidae
Dacnusa sibirica Telenga	Hymenoptera: Braconidae
Dalotia coriaria (Kraatz)	Coleoptera: Staphylinidae
Delphastus catalinae (Horn)	Coleoptera: Coccinellidae
Dicyphus hesperus Wagner	Hemiptera: Miridae
Diglyphus isaea (Walker)	Hymenoptera: Eulophidae
Encarsia formosa Gahan	Hymenoptera: Aphelinidae
Eretmocerus eremicus (Rose)	Hymenoptera: Aphelinidae
Eretmocerus mundus Mercet	Hymenoptera: Aphelinidae
Feltiella acarisuga (Vallot)	Diptera: Cecidomyiidae
Gaeolaelaps gillespieii Beaulieu	Acarina: Laelapidae
Galendromus helveolus (Chant)	Acarina: Phytoseiidae
Galendromus occidentalis (Nesbitt)	Acarina: Phytoseiidae
Goniozus legneri Gordh	Hymenoptera: Bethylidae
Heterorhabditis bacteriophora (Poinar)	Rhabditida: Heterorhabditidae
Heterorhabditis megidis Poinar, Jackson & Klein	Rhabditida: Heterorhabditidae
Heterorhabditis indica Poinar	Rhabditida: Heterorhabditidae

(*Continued*)

TABLE 9.2 (Continued)

Natural enemy species	Order: family
Hippodamia convergens Guérin-Méneville	Coleoptera: Coccinellidae
Leptomastix dactylopii Howard	Hymenoptera: Encyritidae
Macrolophus caliginosus Wagner	Hemiptera: Miridae
Mesoseiulus longipes (Evans)	Acarina: Phytoseiidae
Micromus variegatus (Fabricius)	Neuroptera: Hemerobiidae
Muscidifurax raptor Girault & Sanders	Hymenoptera: Pteromalidae
Muscidifurax raptorellus Kogan & Legner	Hymenoptera: Pteromalidae
Muscidifurax zaraptor Kogan & Legner	Hymenoptera: Pteromalidae
Nasonia vitripennis (Walker)	Hymenoptera: Pteromalidae
Neoseiulus californicus McGregor	Acarina: Phytoseiidae
Neoseiulus cucumeris (Oudemans)	Acarina: Phytoseiidae
Orius insidiosus (Say)	Hemiptera: Anthocoridae
Orius laevigatus (Fieber)	Hemiptera: Anthocoridae
Pediobius foveolatus (Crawford)	Hymenoptera: Eulophidae
Pentalitomastix plethorica Caltagirone	Hymenoptera: Encyrtidae
Peristenus relictus Loan	Hymenoptera: Braconidae
Phytoseiulus persimilis Athias-Henriot	Acarina: Phytoseiidae
Podisus maculiventris Say	Hemiptera: Pentatomidae
Rhyzobius lophanthae (Blaisdell)	Coleoptera: Coccinellidae
Scolothrips sexmaculatus (Pergande)	Thysanoptera: Thripidae
Spalangia cameroni Perkins	Hymenoptera: Pteromalidae
Spalangia endius Walker	Hymenoptera: Pteromalidae
Spalangia nigroaenea Curtis	Hymenoptera: Pteromalidae
Stethorus punctillum Weise	Coleoptera: Coccinellidae
Stratiolaelaps scimitus (Womersley) (=*H. miles*)	Acarina: Laelapidae
Sympherobius barberi Banks	Neuroptera: Hemerobiidae
Tamarixia radiata (Waterston)	Hymenoptera: Eulophidae
Trichogrammatoidea bactrae Nagaraja	Hymenoptera: Trichogrammatidae
Trichogramma brassicae Bezd. (=*T. maidis*)	Hymenoptera: Trichogrammatidae
Trichogramma cacoeciae Marchal	Hymenoptera: Trichogrammatidae
Trichogramma dendrolimi Matsumura	Hymenoptera: Trichogrammatidae
Trichogramma minutum Riley	Hymenoptera: Trichogrammatidae
Trichogramma ostriniae Pang & Chen	Hymenoptera: Trichogrammatidae
Trichogramma platneri Nagarkatti	Hymenoptera: Trichogrammatidae
Trichogramma pretiosum Riley	Hymenoptera: Trichogrammatidae
Thripobius semiluteus Boucek	Hymenoptera: Eulophidae
Steinernema carpocapsae (Weiser)	Rhabditida: Steinernematidae
Steinernema feltiae (Filipjev)	Rhabditida: Steinernematidae
Steinernema scapterisci Nguyen & Smart	Rhabditida: Steinernematidae
Zelus renardii Kolenati	Hemiptera: Reduviidae

Source: LeBeck and Leppla (2021) and Buitenhuis (2014).

17%−91% functional adults were received (number emerged on arrival plus number unable to fly subtracted from the total that emerged). Thus, it appears that the parameters most indicative of product quality in *E. formosa* are quantity received, purity, emergence on arrival, total emergence, and flight capability. Similar results were obtained for *A. colemani*, that is, highly variable product quality from individual sources and between sources, high levels of emergence on arrival in some cases, and emergence of 61%−69% with just above 50% females. Thus, in this study, the six companies delivered an inconsistent number of functional wasps.

Additional quality control studies have been conducted on mass-reared natural enemies, both commercial and governmental, including several *Trichogramma* spp. (Bigler, 1994; Bai et al., 1992; Losey and Calvin, 1995; Hassan and Zhang, 2001; Dias-Pini et al., 2014; González-Cabrera et al., 2014a; St-Onge et al., 2016; Lü et al., 2017; Coelho et al., 2018; Ghaemmaghami et al., 2020), *Splangia cameroni* Perkins (Hymenoptera: Pteromalidae) (Tormos et al., 2011), *Orius insidiosus* (Say) (Heteroptera: Anthocoridae) (Shapiro and Ferkovich, 2002), *Epidinocarsis lopezi* (De Santis) (Hymenoptera: Encyrtidae) (Neuenschwander et al., 1989), *Habrobracon hebetor* Say (Hymenoptera: Braconidae) (Badran et al., 2020), *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) (Veiga et al., 2013) and the mite, *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) (Khanamani et al., 2017). In a study of postshipment product quality control for *A. colemani*, species identification was inaccurate, hyperparasitoids were present in most shipments, an excessive number of wasps emerged before receipt, and a large number of wasps did not parasitize aphids (Fernandez and Nentwig, 1997). The results of quality control tests prior to shipment were much more consistent for a congener, *A. matricariae* (Haliday) (Enkegaard and Reitzel, 1991). The number of wasps produced averaged 1663.1 ± 17.3 (SEM) with 71.9% ± 3.6% emerging and 88.9% flying. Predictable production and synchronized emergence enabled packaging and shipping of an accurate number of wasps that did not emerge prior to receipt. In another study, *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) was evaluated in cooperation with five insectaries to determine the variability of wasp quality (Vasquez and Morse, 2012). All of the insectaries were located in California and the natural enemies were shipped every two months overnight to the University of California, Riverside. Quality assessments included percentage of live parasitoids 1−28 days after receipt, sex ratio, and size of female wasps. Statistically significant variation occurred in all three parameters between insectaries and throughout the year, even though the wasps were reared in controlled environments, for example, mean proportion of females was 0.436−0.591 between insectaries for all shipment dates and 0.393−0.644 between shipment dates for all insectaries during the approximately 2-year period.

9.6 Quality assurance and control data acquisition and analysis

A minimum amount of data is obtained routinely to maintain production, process and product control, and to monitor product utilization and consumer satisfaction. Typical examples of production control data are temperature and relative humidity for all of the rearing environments (Chen et al., 2012). The yield of a developmental stage is typically used for process control, for example, number and proportion of viable eggs, number of larvae per rearing container, the proportion of containers with microbial contamination, etc. Production and postproduction utilization control parameters for natural enemies are quantity, purity, emergence (viability), adult size, sex ratio, flight, fecundity, longevity, and parasitism or predation. For augmentation biological control, customers are satisfied only if an acceptable level of pest population suppression is achieved (Bolckmans, 2003). Therefore, quality assurance data includes both natural enemy characteristics indicative of the quality and sensitivity to change, and feedback from customers. Routine monitoring is expensive and the information is used to make important decisions, so the set of criteria and sampling structure must be limited but adequate. More intense sampling and complicated statistical analyses are needed for supportive research.

A sampling scheme that produces consistent means and standard deviations for each parameter is different for every site-specific rearing system. Variation is minimized by having the same person use standardized techniques to obtain a consistent number of random samples at a prescribed day of the week and time of day. Daily production control data is recorded on electronic data tables and preharvest process control is accomplished by periodically sampling immature insects (Akey et al., 1984). The rearing container is the sampling unit, not the batch or worker shift, and the number of containers sampled at a time depends on their variation. Increased variation among rearing containers maintained in the same environment can indicate a biological problem, whereas variation in batches usually results from inconsistent production or process control. The most efficient indicators of product quality at production and receipt by the consumer are quantity and purity of natural enemies received, percent emergence on arrival, total emergence, and flight capability (Dias-Pini et al., 2014; Pomari-Fernandes et al., 2016). Customers expect shipments to arrive consistently on time, containing the species and number of natural enemies ordered in good condition and at the correct stage of development. The data are analyzed using statistical programs, for example, JMP (SAS Institute Inc., Cary, NC, https://www.jmp.

com) or R (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/), and recorded on quality control charts (Feigenbaum, 1983; Chambers and Ashley, 1984; Wajnberg, 1991, 2003; Leppla, 2009) (Fig. 9.3). The mean and standard deviation for each product characteristic should be plotted over time and stay within the historical range (Chambers and Ashley, 1984), probably within 1−2 standard deviations. This kind of data management also can be applied to feedback from consumers, including both product control and effectiveness parameters. Informed decisions can thereby be made about reducing sources of variation in production and the supply chain.

9.7 Quality assurance system review

Every function of a quality assurance system for mass-reared arthropods should be reviewed periodically to determine if it conforms to established specifications and standards of performance. Depending on the function, reviews can be performed by personnel who accomplish the work, division managers, quality control or some other division, internal professional auditors, upper management, or outside experts. Generally, the complexity, rigor, independence, value, and cost of a review increase along this continuum. It has been argued that reviews within work units are biased or even dishonest but employees who perform the work are most familiar with its nuances. These workers and their division managers will know when "corners are cut," such as circumventing traffic patterns, substituting substandard materials, manipulating check sheets, etc. Typical examples of traffic patterns in arthropod mass-rearing facilities are using doors to avoid inefficient "pass-throughs" or backtracking through contaminated areas to retrieve a piece of equipment. Reviews by members of other divisions or shifts within the same division can be highly biased and overly critical, particularly if any of the reviewers once were assigned to the division or shift being reviewed. It can be enlightening, however, to compare the success of shifts that raise the same arthropods. To the present time, even the largest arthropod mass-rearing facilities have not had internal professional auditors. Upper management often performs reviews but the efforts tend to be superficial unless the managers have remained actively engaged in the quality assurance system. Managers who understand the inner workings of the system tend to have a positive influence on the performance of the functions, whereas inexperienced managers may be fed misinformation and not be taken seriously. Consequently, the least biased and most comprehensive reviews of quality assurance programs for mass-reared arthropods probably have been performed by outside experts.

A periodic review by outside experts begins when it is requested by upper management (Fig. 9.4). The entire quality assurance system is subject to the review and, if possible, is performed when the system is stable and not experiencing a crisis. In practice, however, the resources for a thorough outside review typically become available when there is a major decrease in production or product quality. Reviews conducted during a crisis usually are rushed, lack careful planning, and can be stressful for everyone involved. This makes it difficult for review team members to develop rapport with the employees and earn their trust, avoid unproductive fault-finding, and gain an understanding of possible sources of system failure. Upper management selects review team members with the requisite knowledge, experience, stature, energy, diplomacy, willingness, and sometimes courage to conduct a successful review. Experts in arthropod mass production and utilization who have these attributes are uncommon and may not be available within the required timeframe. Once identified, suitable review team members are empowered to evaluate the system according to explicit, written terms of reference. It is not appropriate to expect a review team to unilaterally identify the details of the review,

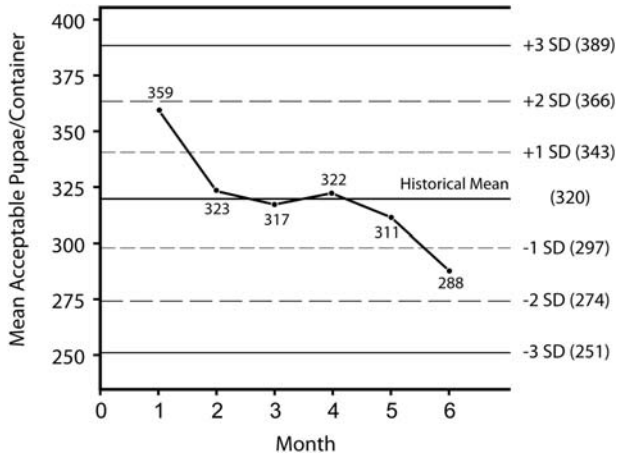

FIGURE 9.3 A quality control chart for the mean monthly number of acceptable fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) pupae per rearing container (data from Leppla and Ashley, 1989). The historical mean was 323.3 (rounded to 320) pupae per container and, using the monthly data, the standard deviation was 22.9. Mean and range charts can be used to monitor any measurable quality assurance parameter.

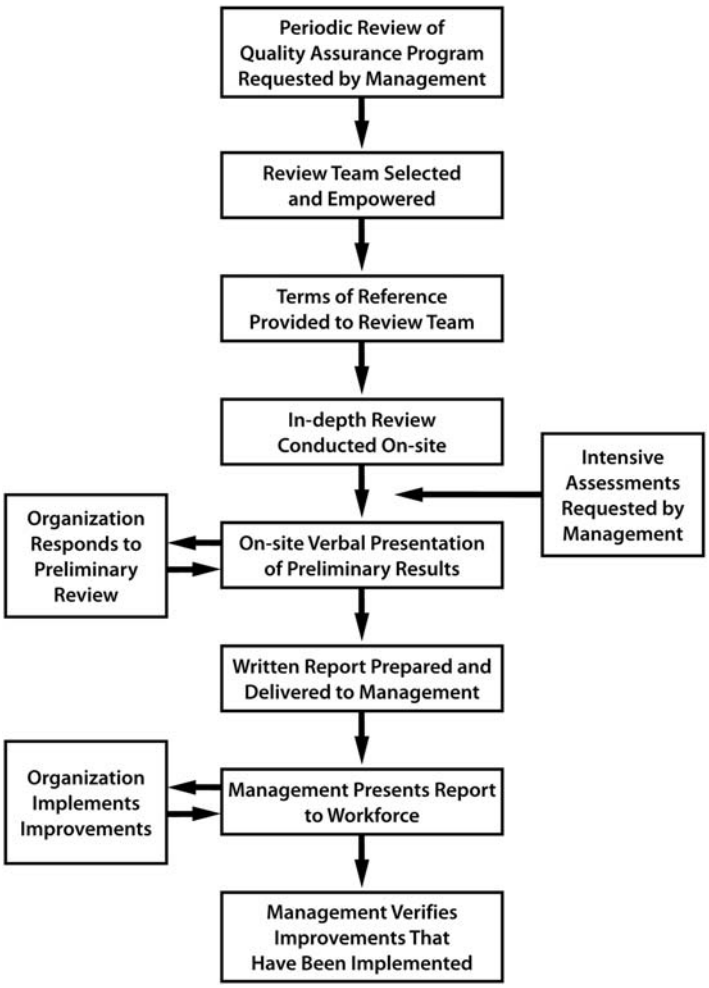

FIGURE 9.4 Process for reviewing a quality assurance system for arthropod mass production based on the experience of the author and Juran et al. (1979).

although they often participate with upper management in refining the goals. The terms of reference and conditions for the review are typically compiled in a document that includes the mission and essential information about every function of the system, available human and physical resources, specific objectives for the review, reporting requirements, the timeframe, and associated expectations. The entire quality assurance system likely will be evaluated for ways to make it more reliable and cost-effective but this charge is too general. A more specific goal, for example, would be to evaluate every step in the product supply chain to identify causes for delays, so that high quality 2-day-old natural enemies can be delivered to customers consistently. In consultation with management and selected employees, the review team can develop actions intended to achieve the goals of the review.

While the in-depth quality assurance system review is underway, the review team members delve into the nuances of each function and periodically consult with management to refine the process. Managers may request that intensive evaluations be made concerning particular aspects of the system. They may have a special interest or perceive that a process requires an especially high level of scrutiny. This often occurs when a new piece of equipment is installed to automate a rearing procedure, for example, a form-fill-seal machine to automate packaging and infestation of a diet. Adjustments invariably are made as the workers and arthropods adapt to the change. As the review progresses, preliminary results are assembled and analyzed by the review team members and, if gaps exist, additional information is obtained. The time required to reflect on the findings and complete a comprehensive review is another reason for not conducting it when the system is failing and requires rapid restoration. A verbal report is presented on-site before the review is completed to explain preliminary findings and obtain feedback from the workforce. Care is taken to identify strengths and opportunities while avoiding the impression that there are major deficiencies and a need for individual employees to improve. The final written report is delivered to management within a few weeks, enabling managers to present it to the workforce expeditiously. Considerable discussion and detailed analysis of the findings by the managers

and employees take place before deciding to implement specific recommendations for improving the system. Feedback is then provided to the review team on each point in the report, including plans to implement changes as well as findings for which there is disagreement. The changes can be made either to restore or enhance specific aspects of the quality assurance system. Finally, management verifies the improvements that have been made and frequently asks a particularly insightful review team to remain available for consultation or a subsequent review.

The following outline for reviewing a quality assurance system for mass rearing arthropods and reporting the results was adapted initially from a World Wildlife Fund project audit framework, updated as the "WWF Standards of Conservation Project and Programme Management (PPMS) Version: 30 June 2017." (http://www.panda.org/standards/evaluations_terms_of_reference/):

9.7.1 Approach

- Purpose and scope of the quality assurance system review;
- Specific objectives for the quality assurance system review;
- Review team members, qualifications, interest in review, and roles;
- System functions being reviewed for performance;
- Expected outputs and outcomes for the review;
- Methodology and timeline, data collection and analysis, and ethics and fairness.

9.7.2 Review of functions (successes and failures)

- System design, quality and effectiveness;
- Research and methods development support;
- Production, process and product control data;
- Utilization control data;
- Communication and feedback mechanisms;
- Problems encountered that require urgent attention.

9.7.3 Conclusions

- Reasons for successes and failures of system functions;
- Insights into the findings;
- Innovations for improving the quality assurance system.

9.7.4 Recommendations (based on evidence and insights)

9.7.4.1 Attachments to report

- Terms of reference for the review;
- Short biographies of the evaluators;
- Structure and timetable for the review;
- List of individuals interviewed;
- List of supporting documentation reviewed;
- Research instruments, questionnaires, and interview guides;
- Specific data acquired and summary tables.

The World Wildlife Fund audit framework has more detail, along with a template for the terms of reference that includes the project background and context, purpose and objectives of the review, audience for the review, review issues and key questions (review matrix), quality and relevance of design, effectiveness, efficiency of planning and implementation, impact, potential for sustainability, replication and magnification, methodology, profile of the review team, evaluation timetable, cost, and logistical support.

9.8 Research on quality assessment for mass-reared parasitoids and predators

Virtually every aspect of augmentative biological control technology can be improved, including the feasibility of developing and marketing new natural enemy products, producing them reliably and efficiently, applying and evaluating them, and modifying the environment in which they are released (Table 9.3). A product is developed only if research has determined that the natural enemy is potentially effective, there is an identified market or need for the product, it can be produced and delivered to meet the demand, and an associated quality assurance system can be designed and implemented. Research in both the laboratory and field is needed before mass-reared parasitoids and predators can reach their potential applications in integrated pest management (Glenister and Hoffman, 1998; van Lenteren and Tommasini, 1999; Rendon et al., 2006).

Research on improving the quality and effectiveness of mass-produced natural enemies should be conducted collaboratively by entomologists and commercial producers and suppliers who have considerable experience in marketing biological control products and determining their cost/benefit (Parra, et al., 2015; Mhina et al., 2016). The commercial biological control industry lacks the resources and often expertise to make significant improvements in rearing, packaging, storing, shipping, applying, and evaluating their products. Consequently, researchers should assist the industry by conducting quality assurance studies extending from feasibility through mass production and utilization in the application environment, thus assuring that their discoveries are evaluated and possibly adopted. Researchers could assess product quality collaboratively and report the results to the supplier immediately so that problems can be identified quickly and corrected in the production and distribution chain. Product control assessments conducted by the recipient can indicate that a problem exists but not identify the cause (Webb et al., 1981; Steward et al., 1996; O'Neil et al., 1998; Thomson and Hoffmann, 2002; Vasquez et al., 2004; Vasquez and Morse, 2012). Possible causes include changes in hosts or diets (Rodriguez et al., 2002; Lopez et al., 2009), rearing materials and environments, personnel and their training, handling and packaging, transportation and routing, treatment on receipt, and testing procedures. These kinds of changes affect colonization and colony maintenance and can result in genetic bottlenecks that reduce survival and heterozygosity (McMurtry and Scriven, 1975; Jones et al., 1978; Bartlett, 1984, 1985; Joslyn, 1984; Mangan, 1992;

TABLE 9.3 Research to advance quality assurance for mass-produced natural enemies.

Type of research	Research priorities
Feasibility/market analysis	Natural enemy effectiveness in controlling target pests Product development Quality assurance systems
Mass production	Rearing facilities Mechanized equipment Rearing materials Natural and artificial diets Rearing and harvesting techniques Production control Process control Product quality control
Utilization	Product handling and storage Automated counting technology Improved packaging and shipping Extended use period Efficient scouting techniques Optimal release numbers, rates and timing Mechanized application technologies Efficient product evaluation procedures Utilization control
Application environment	Site-specific pest prevention Systems to maximize the effectiveness of natural enemies Natural enemy use with pesticides Systems with multiple products and pests Effectiveness in seasonal and perennial crops

Mohaghegh et al., 1998; Ode and Heinz, 2002; Hegazi and Khafagi, 2005; He and Wang, 2006; Gonzalez et al., 2007; Sarvary et al., 2008; Joyce et al., 2010). The goal is to produce natural enemies with characteristics that approximate those of the colony founders and can be maintained indefinitely (Messing et al., 1993; Prezotti et al., 2004). To minimize changes, producers establish standard protocols for production and product evaluation that specify taxon, sampling scheme, replication, materials, procedures, data analysis, and requirements for reporting. Once a standardized evaluation protocol is established for a product, it also can be used by both trained suppliers and customers.

Typically, entomologists and agricultural engineers jointly design rearing facilities and equipment, select materials, and establish an arthropod production line (Harrell and Gantt, 1984). They also collaboratively conduct postproduction research on handling, release and evaluation of natural enemy products that could significantly increase their acceptance in the marketplace. The cost of natural enemy products could be reduced by designing automated counting, packaging, storage, transportation, and application equipment (Pearson et al., 2002). Also, storage and stockpiling of natural enemies would enable suppliers and customers to better time applications. Cold storage has the potential for stockpiling parasitoids (Leopold, 2000, 2007; Tezze and Botto, 2004; Chen et al., 2011; Sun et al., 2017; Benelli et al., 2018; Rezaei et al., 2020; Zhang et al., 2020) and predators (Niijima et al., 2002; Luczynski et al., 2008; Ghazy et al., 2012; Maes et al., 2012; Bueno et al., 2014; Sakaki et al., 2019). However, prolonged storage can reduce the quality of natural enemies (Rathee and Ram, 2018). For *A. aphidimyza*, stored females had low fecundity and therefore did not produce the number of progeny needed for successful biological control (Fernandez and Nentwig, 1997). Females that had not been stored were extremely useful in controlling a wide range of aphid species, whereas those that had been stored for months had little impact (Luczynski et al., 2007). Adult emergence could be increased by holding *A. aphidimyza* pupae in a transparent package with high humidity, enabling the midges to be observed and removed. The effectiveness of natural enemies shipped over long distances also could be improved through research in packaging, for example, durability, insulation, and monitoring requirements. Some natural enemy products are packaged in substrates that facilitate mechanical application and these "carriers" also could be improved.

Entomological research is needed to advance natural enemy mass-rearing systems, especially to develop more efficient natural and factitious hosts and practical artificial diets. (Reis et al., 2003; Wackers and van Lenteren, 2003; Arijs and De Clercq, 2004; Nathan et al., 2006; Vandekerkhove et al., 2006; Bonte and De Clercq, 2011; Bonte et al., 2011) and alternative rearing techniques (Bonte and De Clercq, 2010). A parasitoid typically has higher fecundity and survival on a natural host, whereas a factitious host can be much less expensive and more practical to use for mass production (Watson et al., 2000; Ramalho and Dias 2003; Bonte and De Clercq, 2008; Tuncbilek et al., 2009). Considerable research has been conducted on factitious hosts for parasitods (Bergeijk et al., 1989; Rojas et al., 1998; Grenier and De Clercq, 2003; Ramalho and Dias, 2003; Saadat et al., 2016; Glenister et al., 2018; Abdi et al., 2020; Konopka et al., 2020; Zhou et al., 2020) and alternative prey for predators (Watson et al., 2000; Silva et al., 2013; Queiroz et al., 2017; Riahi et al., 2017, 2018; Montoro, et al., 2020; Abbas et al., 2020; Toft et al., 2020). Host eggs can be stored under cold conditions to prolong their usefulness for rearing natural enemies (Chen and Leopold, 2007; Mahmoud and Lim, 2007; Tuncbilek et al., 2009; Spinola-Filho et al., 2014; Cingolani et al., 2015; Edwin et al., 2016). However, it may not be possible to use cold-stored larvae to rear parasitoids (Pastori, et al., 2013). Irradiated Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) larvae have been used successfully to rear the parasitoid, *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Ichneumonidae) (Viscarret et al., 2012; Carta Gadea et al., 2020). *Coptera haywardi* (Ogloblin) (Hymenoptera: Diapriidae) has been reared on irradiated Mediterranean fruit fly pupae (Núñez-Campero et al., 2020). Artificial diets continue to be developed for predaceous larvae and adult arthropods (Arijs and De Clercq, 2004; Vandekerkhove et al., 2006; Bonte and De Clercq, 2011; Bonte et al., 2011; Liu and Zeng, 2014; Ali et al., 2016; Rojas et al., 2016; Sun et al., 2018) and some have been microencapsulated (Tan et al., 2013; Tan et al., 2015). Artificial diets for natural enemies have been formulated with gelling agents (Neuenschwander et al., 1989) and diet-filled eggs were developed to rear *Trichogramma* spp. (Nordlund, et al., 1997; Lü et al., 2017). The quality of parasitoids also can be affected by artificial diets used to rear their hosts (Reis et al., 2003; Nathan et al., 2006; Wetro et al., 2014; Farahani et al., 2016; Ras et al., 2017; Moghaddassi et al., 2019; Pascacio-Villafán et al., 2020; Pezzini et al., 2020). The longevity of adult predators (Fratoni et al., 2020) and parasitoids (Wackers and van Lenteren, 2003; Benelli et al., 2017; Gonçalves et al., 2016) often can be extended by feeding them concentrated carbohydrate solutions, supplemented with pollen (Picciau et al., 2019).

Mass-reared natural enemies are most effective if applied in site-specific pest management systems, those that conserve beneficial organisms and other natural controls (Perez-Alvarez et al., 2019). However, natural enemies often are evaluated and applied inappropriately in cropping systems and other situations designed to optimize the use of pesticides for maintaining pests below acceptable thresholds. In these systems, natural enemies generally are not compatible with pesticides and their use is precluded (Hall and Nguyen, 2010). After being released, natural enemies must survive

and hopefully reproduce (Sandanayaka et al., 2018). Research is needed on the effectiveness of natural enemies, and the economic and ecological limits of their use in biological control (Collier and Van Steenwyk, 2004). Research also is needed to determine the immediate and residual toxicity of chemical pesticides to natural enemies, so that incompatibilities can be predicted and avoided (Pree et al., 1989; Beers et al., 2016). Increasingly, pest management systems are designed to maximize the effectiveness of parasitoids and predators in concert with compatible pesticides (El-Wakeil et al., 2013). In these kinds of systems, most pesticides are not acceptable because of their nontarget effects, frequency of application, and long-term higher cost. Research also is needed to determine and demonstrate how natural enemies can be used in cropping systems and monitor their effectiveness (Masry et al., 2019) based on scouting and reasonable damage thresholds. Pest management can be complicated further when multiple parasitoids and predators are released to manage several pest species. Ultimately, reliance on natural enemies results from research that demonstrates their effectiveness in significantly reducing pest populations (Li et al., 2014; Yang et al., 2014; Martínez et al., 2018). According to Penn et al. (1998), "If biologically intensive integrated pest management is to become a predominant practice, and if a substantial increase in the use of mass-reared arthropods and other biologically based products is to be realized, the entire agricultural community (academia, government, growers and the pest control industry) must work together to develop comprehensive quality assurance programs and to specifically design pest management systems to accommodate these products."

9.9 Conclusion

This chapter on concepts and methods of quality assurance for mass-reared parasitoids and predators extends a previous book chapter, "The basics of quality control for insect rearing" (Leppla, 2009) and this chapter in the first edition of "Mass Production of Beneficial Organisms" (Leppla, 2014). Here, emphasis is placed on mass rearing arthropod natural enemies, and the concept of total quality control in insect mass production has been expanded to emphasize customer involvement and satisfaction. This expansion combines all aspects of developing, producing, delivering, using, and evaluating natural enemy products. Mass production of high-quality arthropods requires a well-designed and managed system that is efficient and reliable. To maintain reliability, every function of the system should be reviewed periodically so that it continues to conform to established standard operating procedures. Key determinants of quality are assessed to ensure the performance of mass-produced arthropods and to detect changes that could compromise their effectiveness in the field (Benedict et al., 2009).

The purpose of mass-rearing beneficial arthropods is to satisfy customer needs and make a profit if the products are marketed. Therefore, a product must be effective, marketable, deliverable to meet demand, and produced and used according to an associated quality assurance system. Before investments are made in developing an arthropod product, a comprehensive business plan should be developed that includes these provisions and the feasibility of the integrated pest management program that requires high-quality natural enemies or a market analysis for commercial ventures. It is essential to have customers involved in determining their needs and expectations and drafting associated product specifications and standards. Producers and suppliers not only make sure that the customers are satisfied but also deliver high-quality products based on appropriate assessments. This can be challenging if there is a lack of consistent rearing and predictable performance of natural enemy products. In the absence of customer feedback that identifies the cause of unacceptable product quality, producers and suppliers typically resupply the purchased product and provide technical support in an attempt to assure that it ultimately is effective. Increased quantity does not compensate for poor quality, however. Natural enemies will continue to be produced and used in greater quantities if they are cost-effective and suppress pest populations to levels acceptable to the customers.

An arthropod mass production and utilization system designed and operated to maximize product quality and effectiveness include the following divisions: management, personnel, methods development, research, material, production, quality control, and utilization. Management is to fully participate in planning the organization, instituting policies for its operation, establishing standard operating procedures and schedules, assuring the competency of employees, and providing ongoing administrative support for the entire system. Employees must be appropriate, well trained, and conscientious in performing their assigned tasks, and periodically evaluated and rewarded. The methods development division is responsible for increasing product quality and reducing costs by optimizing the system, often by pilot testing potential advancements from research. Applied research is conducted to improve colonization, production, quality control, and utilization. The material division verifies that specifications and standards are met for all materials and supplies purchased from vendors. Arthropod mass production requires diligent adherence to standard operating procedures and immediate correction of deviations through production control. The quality control division monitors rearing processes and products, providing rapid feedback to production and management. Feedback directly to the production division

enables immediate problems to be corrected. Management may use quality control feedback to make long-term changes to the system. The utilization division conducts product quality control assessments at key points along the supply chain to maintain product effectiveness and assure customer satisfaction.

Product quality standards and associated tests have been developed for many of the more commonly produced predators and parasitoids. A set of tests can be selected and adapted for use in specific situations to measure characteristics indicative of product quality and consistency. Depending on the species, pre- and postshipment product quality control assessments could include the number, identity and stage of insects supplied and their emergence, sex ratio, survival, and reproduction. Movement and hyperparasitism also are determined for some species. The data can be analyzed using simple statistics and recorded as means and standard deviations on quality control charts. Tests are available for more than 28 species of insects and mites (Buitenhuis, 2014). These tests have indicated that the quality of natural enemies is highly variable between insectaries and throughout the year, possibly because of limited employee experience and turnover. To reduce this variability, producers, suppliers and customers should routinely conduct the same set of accurate assessments and rapidly share the results. A minimum amount of monitoring is required to maintain production, process, product, and utilization control.

The quality of mass-produced natural enemies is improving incrementally due to advances in associated research and technology. Considerably more research is needed, however, to establish quality assurance systems that extend from feasibility through mass production to application and evaluation. Field studies to assure the peak performance of natural enemies are a high priority. Molecular techniques can be used to rapidly identify species (González-Cabrera et al., 2014b) and strains, screen colonies for pathogens and hyperparasites, and eventually genetically engineer natural enemies (Hoy, 1979). Research is required to produce natural enemies on alternative hosts and design and build reliable field insectaries. Entomologists, engineers, food technologists, and others could formulate, prepare, and package better diets, evaluate rearing materials and environments, increase automation of rearing processes, test alternative transportation options, and help train personnel. Postproduction research on handling, release and evaluation of natural enemy products is crucial and researchers, suppliers, and customers could jointly develop efficient product quality control assessments. Mass-reared natural enemies must be applied in integrated pest management systems designed to maximize their effectiveness, so research should be conducted on compatible technologies and practices. New selective pesticide chemistries could provide opportunities to combine biological and chemical control, especially when multiple pests are present or a backup is needed for the natural enemies. Since there are no pesticide-like labels for predators and parasitoids, it would help to have biological control industry guidelines for using each species that would specify target pests and minimize errors in handling, application rates and timing. Thus, research is needed on almost every aspect of quality assurance for mass-produced arthropods to design and operate systems that produce and deliver products that satisfy customer expectations.

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Section II

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Chapter 10

Production of entomopathogenic nematodes

David I. Shapiro-Ilan[1], Luis Garrigós Leite[2] and Richou Han[3]
[1]USDA-ARS, Southeastern Fruit and Tree Nut Research Unit, Byron, GA, United States, [2]Institute of Biology, APTA, São Paulo, SP, Brazil, [3]Guangdong Entomological Institute, Guangzhou, P.R. China

10.1 Introduction

The objective of this chapter is the review and analysis of methodology for the production of entomopathogenic nematodes (EPNs). Nematodes are round worms (also known as eelworms or threadworms) of the phylum Nematoda. EPNs have been defined as parasitic nematodes that are mutualistically associated with bacterial symbionts; all life stages of the nematode, except for the dauer stage (see below) are found exclusively inside the insect host (Grewal et al., 2005; Onstad et al., 2006). Historically this definition of EPNs has referred to the families Steinernematidae and Heterorhabditidae. Recently, the definition of EPNs was expanded to include other nematodes such as certain species of the genus *Oscheius* (Dillman et al., 2012a). Nonetheless, this chapter will focus exclusively on the genera *Heterorhabditis* Poinar, and *Steinernema* Travassos because they are the only EPNs for which mass production methods have been developed, and they are the only ones sold commercially for biocontrol purposes.

More than 129 species of steinernematids and heterorhabditids have been described to date (at least 106 steinernematids and 23 heterorhabditids) (Stock and Hunt, 2005; Shapiro-Ilan et al., 2018; Bhat et al., 2020). The relationship between these nematodes and their bacterial symbionts is highly specific; bacteria from the *Xenorhabdus* spp. And *Photorhabdus* spp. Are associated with *Steinernema* and *Heterorhabditis*, respectively (Poinar, 1990; Griffin et al., 2005; Lewis and Clarke, 2012). The bacteria are the primary agents responsible for killing the insect host and provide the nematodes with a source of nutrition. The nematodes are also critical to the symbiotic relationship because of the bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. (that are associated with nematodes used in biocontrol) depend entirely on the nematode to vector them from one insect host to another; additionally, the nematodes assist in suppressing the host immune system (Dowds and Peters, 2002; Lewis and Clarke, 2012).

A generalized life cycle of EPNs is depicted in Fig. 10.1. The infective juvenile (IJ) nematode, or "dauer" stage (Fig. 10.2), which is the only free-living stage, enters the host via natural openings, that is, mouth, anus, spiracles, or occasionally through the insect cuticle; in heterorhabditids, penetration through the cuticle may be facilitated by a specialized "tooth" structure (Dowds and Peters, 2002). After entering the insect's hemocoel, IJs release their symbiotic bacteria and the host dies usually within 24−72 hours. Once inside the host, the developmentally arrested IJs reinitiate their lifecycle and molt to fourth-stage juveniles (the reinitiation of the lifecycle is referred to as "recovery"). The nematodes feed on multiplying bacteria and host tissues while completing their development (having six life stages: egg, four juvenile stages, and adult); the nematodes may carry out one to three generations within the host (Lewis and Clarke, 2012). When the nutritive value in the host becomes depleted, IJs exit from the insect cadaver searching for new hosts (Lewis and Clarke, 2012). The heterorhabditid lifecycle differs from that of the steinernematid in that the first generation of heterorhabditids are exclusively hermaphrodites and subsequent generations include males, females, and hermaphrodites (Strauch et al., 1994; Koltai et al., 1995). In contrast, all steinernematid species possess only amphimictic forms (males and females), except one species that (similar to heterorhabditids) was found to also possess hermaphrodites in the first adult generation, that is, *Steinernema hermaphroditum* Stock, Griffin, and Chaerani (Stock et al., 2004).

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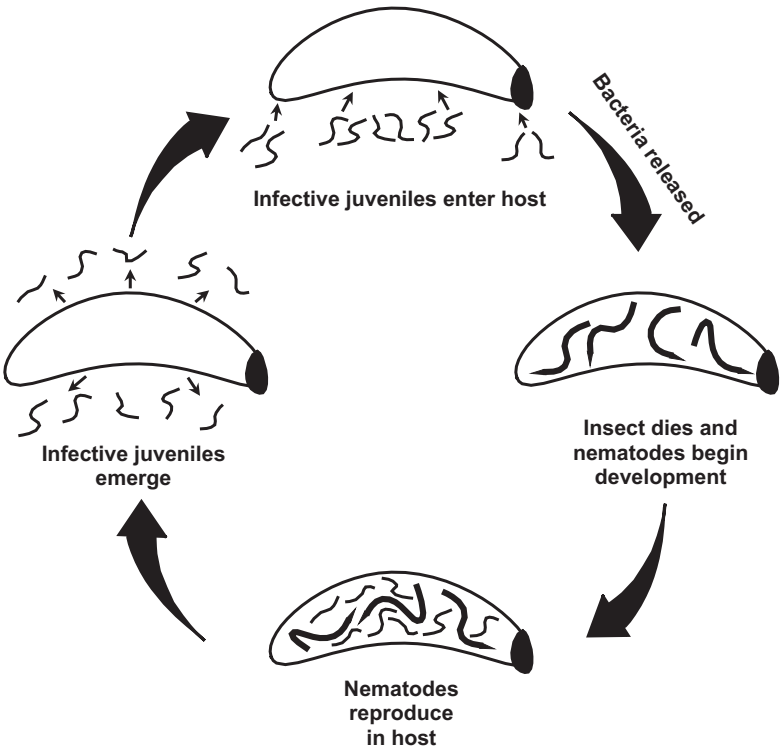

FIGURE 10.1 A generalized life cycle of entomopathogenic nematodes.

FIGURE 10.2 An infective juvenile steinernematid nematode.

EPNs possess many positive attributes as biological control agents for the suppression of insect pests (Shapiro-Ilan and Grewal, 2008; Shapiro-Ilan et al., 2012a, 2018). They are safe for humans and are generally safe for other nontarget organisms and the environment (Akhurst and Smith, 2002; Ehlers, 2005). The level of safety associated with EPNs has led to a lack of pesticide registration requirements in many countries such as the United States and nations in the European Union (Ehlers, 2005). Furthermore, EPNs have a wide host range (with few exceptions) and kill the target host relatively rapidly (Lewis and Clarke, 2012; Shapiro-Ilan et al., 2012a).

Based on the attractive attributes described above, EPNs have been developed as biocontrol agents on a commercial level. They are currently being produced by more than ten companies in Asia, Europe, and North America, and, to date, at least thirteen different species have reached commercial development: *Heterorhabditis bacteriophora* Poinar, *H. indica* Poinar, Karunakar and David, *H. marelata* Liu and Berry, *H. megidis* Poinar, Jackson and Klein, *H. zealandica*

Poinar, *Steinernema carpocapsae* (Weiser), *S. feltiae* (Filipjev), *S. glaseri* (Steiner), *S. kushidai* Mamiya, *S. kraussei* (Steiner), *S. longicaudum* Shen and Wang, *S. riobrave* Cabanillas, Poinar and Raulston, and *S. scapterisci* Nguyen and Smart (Lacey et al., 2001; Georgis et al., 2006; Kaya and Stock, 1997: unpublished). EPNs can suppress a wide variety of economically important pests, many of which are targeted commercially (Table 10.1, also see Shapiro-Ilan and Gaugler, 2002; Grewal et al., 2005; Georgis et al., 2006; Lacey and Georgis, 2012; Shapiro-Ilan et al., 2018).

The efficacy of EPNs in suppressing insects depends on selecting the appropriate nematode for the target pest (Shapiro-Ilan et al., 2002b). A suitable candidate must possess a high level of virulence (disease-causing power) toward the host. The nematode must be able to evade host defenses and initiate pathogenesis. The ability of EPNs to persist in the environment may also contribute to host suitability and biocontrol efficacy. Indeed, in some cases enhanced persistence may even compensate for lower virulence. However, high levels of efficacy following EPN applications generally persist only for two to six weeks. Thus, EPNs are usually applied in an inundative or "pesticidal" manner where little or no recycling is expected and re-application is required annually or seasonally. Nevertheless, there have also been some reports of prolonged pest control over several seasons or years (Shapiro-Ilan et al., 2002b).

Nematode persistence depends on the nematode species or strain, host density, and most importantly environmental factors (e.g., temperature, moisture, etc.) (Shapiro-Ilan et al., 2006). EPNs are highly sensitive to desiccation and ultraviolet light. Therefore, applications made to soil or other cryptic habitats (and made during the early morning or

TABLE 10.1 Insects pests targeted for control using entomopathogenic nematodes.[a]

Common name	Scientific name	Nematode(s)[b]
Artichoke plume moth	*Platyptilia carduidactyla* (Riley)	Sf, Sc
Banana moth	*Opogona sachari* Bojer	Hb, Sc
Banana root borer	*Cosmopolites sordidus* (Gemar)	Sc, Sf, Sg
Billbug	*Sphenophorus* spp.	Sc
Black cutworm	*Agrotis ipsilon* (Hufnagel)	Sc, Hb
Black vine weevil	*Otiorhynchus sulcatus* (F.)	Hb, Hmeg, Sk
Blue green weevils	*Pachneus* spp.	Sr, Hb
Borers	*Synanthedon* spp. (Lepidoptera: Sesiidae)	Sc, Hb, Sf
Cabbage maggot	*Delia radicum* (L.)	Sf
Cat flea	Ctenocephalides felis (Bouché)	Sc, Hb
Chinch bug	*Blissus leucopterus* (Say)	Sc
Codling moth	*Cydia pomonella* (L.)	Sf, Sc
Corn rootworm	*Diabrotica* spp.	Hb, Sf
Cranberry girdler	*Chrysoteuchia topiaria* (Zeller)	Sc
Diamondback moth	*Plutella xylostella* (L.)	Sc
Diaprepes root weevil	*Diaprepes abbreviatus* (L.)	Sr, Hb, Hi
Fungus gnats	Diptera: Sciaridae	Sf, HbHd, Sc, Sf
Large pine weevil	*Hylobius abietis* (L.)	
Leafminer	*Liriomyza* spp. (Diptera: Agromyzidae)	Sf, Sc
Mole crickets	*Scapteriscus* spp. (Orthoptera: Gryllotalpidae)	Ss, Sr, Sc
Navel orangeworm	*Amyelois transitella* (Walker)	Sc
Pecan weevil	*Curculio caryae* (Horn)	Sc
Plum curculio	*Conotrachelus nenuphar* (Herbst)	Sr
Small hive beetle	*Aethina tumida* (Murray)	Sr, Hi
Strawberry root weevil	*Otiorhynchus ovatus* (L.)	Hb, Hmar
Sweetpotato weevil	*Cylas formicarius* (F.)	Hb, Hi
Western flower thrips	*Frankliniella occidentalis* (Pergande)	Sf
White grubs	Coleoptera: Scarabaeidae	Hb, Hmeg, Hmar, Sg

[a]*This table includes major target pests and the nematodes used to control them based on industry recommendations and refereed scientific articles indicating high levels of efficacy; the table is not meant to be an exhaustive list (see also Grewal et al., 2005; Georgis et al., 2006; Lacey et al., 2001; Shapiro-Ilan et al., 2018).*

[b]*Hb,* Heterorhabditis bacteriophora*; Hd,* H. downesi *(Stock, Burnell and Griffin); Hi,* H. indica*; Hmar,* H. marelatus*; Hmeg,* H. marelatus*; Sc,* Steinernema carpocapsae*; Sf,* S. feltiae*; Sg,* S. glaseri*; Sk,* S. kraussei*; Sr,* S. riobrave*; Ss,* S. scapterisci.

evening) tend to be most successful. Temperature extremes (below 1°C and above 35°C) can be detrimental to nematodes. The optimum temperature for maximum efficacy depends on nematode species or strain; some nematodes are relatively more heat-tolerant such as *H. indica*, and *S. riobrave*, whereas others are relatively more cold-tolerant, for example, *H. megidis* and *S. feltiae*.

The suitability of an EPN to control a particular target pest may also depend on the nematode's foraging strategy (Shapiro-Ilan et al., 2002b; Lewis and Clarke, 2012). Foraging strategies exhibited by EPNs exist along a continuum from ambushers to cruisers. Ambushers tend to use a sit and wait for strategy, that is, they usually stand on their tails (nictating) and wait until a host comes close. Cruisers generally actively seek out their hosts and cue into certain target volatiles prior to contacting the host. Examples of nematodes that exhibit foraging behavior characteristic of ambushers include *S. carpocapsae* and *S. scapterisci*, those exhibiting behavior typical of cruisers include *H. bacteriophora*, *H. megidis*, and *S. glaseri*, and those with intermediate search behaviors include *S. feltiae* and *S. riobrave*. Ambushers may be most successful at infecting mobile insects on or near the soil surface whereas cruisers can be most successful at infecting sessile insects below the soil surface. Despite these generalities, some ambusher nematodes have been quite effective in suppressing belowground pests (Shapiro-Ilan et al., 2009a; Dillon et al., 2007).

Research in the field of entomopathogenic nematology has advanced substantially in recent years in a variety of subject areas including biological control (Grewal et al., 2005; Shapiro-Ilan et al., 2006; Lacey and Georgis, 2012; Dolinski et al., 2012), ecology (Lewis et al., 2006; Campos-Herrera et al., 2012), bioactive metabolites (Bode, 2009), symbiosis (Goodrich-Blair and Clarke, 2007), and genetics (Ciche, 2007; Dillman et al., 2012b). Research on improving mass production has also advanced (Shapiro-Ilan et al., 2012b). EPNs are currently produced using in vivo or in vitro (solid and liquid culture) methods (Friedman 1990; Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan et al., 2012b). Each approach has its advantages and disadvantages in terms of production cost, technical expertise, the economy of scale, and quality of the end product. Following production, a variety of formulations are available to facilitate application (Grewal, 2002). We provide a summary of EPN production methods, factors that affect efficiency, and methods for improvement as well as future research directions needed.

10.2 In vivo production

10.2.1 Basic method

Methods for in vivo production of EPNs have been reported by various authors (Dutky et al., 1964; Poinar, 1979; Woodring and Kaya, 1988; Lindegren et al., 1993; Flanders et al., 1996; Kaya and Stock, 1997; Shapiro-Ilan et al., 2002a, 2014, 2016). A step-by-step methods description of basic and advanced in vivo production methods is depicted in Shapiro-Ilan et al. (2016). Generally, the approach is based on a two-dimensional system that relies on production in trays and shelves (Friedman, 1990; Shapiro-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan et al., 2012b, 2016). These systems usually revolve around the concept of a White trap (White, 1927), which is a device used for harvesting IJs that takes advantage of the progeny IJ's natural migration away from the host cadaver upon emergence. The White trap consists of a dish or tray on which the cadavers rest; the dish is surrounded by water, which is contained by a larger arena (Fig. 10.3).

The in vivo approach consists of inoculation, harvest (via White trap), concentration, and (if necessary) decontamination. Inoculation is achieved by applying IJs on a dish or tray containing the host insects and lined with absorbent

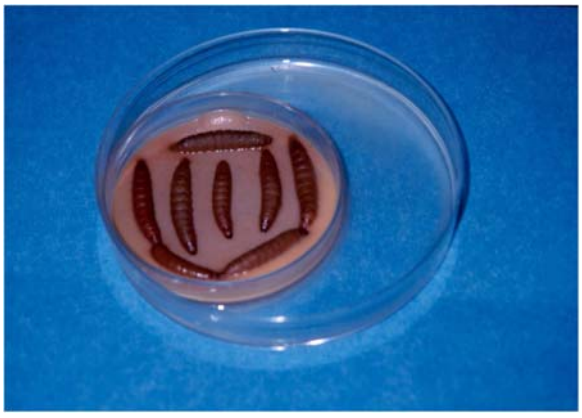

FIGURE 10.3 A White trap. The area around the smaller Petri dish is filled with water below the level of dish. Nematode progeny crawl over the small dish lid into the water trap.

paper or another material conducive to nematode infection such as soil, cheesecloth, or plaster of Paris. After approximately 2–5 days postinoculation, infected insects are transferred to the White traps. It is important not to allow the infections to progress too long before the transfer, otherwise, the chance of the cadavers rupturing, or harm to reproductive nematode stages, will increase (Shapiro-Ilan et al., 2001). The IJs are harvested after they emerge and migrate into the surrounding water trap. The scale of the White trap in size and number can be expanded to commercial levels, for instance, trays as large as 1 m^2 are conceivable as long as mechanisms for the IJs to enter the water trap are available. Following harvest, the concentration of nematodes can be accomplished by gravity settling, for example, in large cone-shaped vessels, and or vacuum filtration (Lindegren et al., 1993). Gravity settling is simple and straightforward, but one potential drawback is that prolonged lack of aeration can be detrimental to the nematodes. Centrifugation is also feasible (Kaya and Stock, 1997), but, for commercial in vivo operations, the capital outlay for a centrifuge of sufficient capacity may be unwarranted.

10.2.2 Factors affecting efficiency

The efficiency of production is derived from nematode yield relative to cost outputs. The primary expenses for in vivo production include the costs of insect hosts and labor. Methods to reduce these costs will be discussed in the subsequent section. Factors affecting nematode yield in in vivo production include the nematode and host species, inoculation parameters, and environmental conditions.

To some extent, variation in yield among nematode species is inversely proportional to IJ size (see Grewal et al., 1994 and Hominick et al., 1997), though some species simply have innately high reproductive capacities (such as *H. indica* and *S. riobrave*). For example, yields of *S. riobrave* (average body length of IJ = 622 mm) may exceed 300,000 IJs per insect in *Galleria mellonella* L. whereas for *S. glaseri* (average body length of IJ = 1133 mm) yields do not exceed 50,000 IJs in the same host (Grewal et al., 1994; Stock and Hunt, 2005). Thus, the choice of nematode species must be taken into account when developing in vivo production ventures. However other considerations may also be critical in choosing which nematode(s) to produce such as matching the appropriate nematode species to the selected target pests, environmental conditions of the target site, and marketing considerations (Shapiro-Ilan et al., 2002b).

The choice of insect host is also important to maximize yields and efficiency in production. Due to its high susceptibility to most nematodes, ease in rearing, wide availability, and ability to produce high yields, the last instar of the greater wax moth, *G. mellonella*, is the most common insect host used for in vivo laboratory and commercial EPN production (Woodring and Kaya, 1988). Only a few EPNs exhibit relatively poor reproduction in *G. mellonella* (due to extremes in host specificity), for instance, *S. kushidai* and *S. scarabaei* Stock and Koppenhöfer appear to be especially adapted to hosts in the family Scarabaeidae (Order = Coleoptera) and *S. scapterisci* appears to be specially adapted to the order Orthoptera (Mamiya, 1989; Nguyen and Smart, 1990; Kaya and Stock, 1997; Grewal et al., 1999; Koppenhöfer and Fuzy, 2003). Significant research and commercial application have also been achieved for the production of EPNs in *Tenebrio molitor* L. (Blinova and Ivanova, 1987; Shapiro-Ilan et al., 2002a). Various other hosts have been studied for in vivo nematode production including the navel orangeworm, *Amyelois transitella* (Walker), tobacco budworm, *Heliothis virescens* (F.), cabbage looper, *Trichoplusia ni* (Hübner), pink bollworm, *Pectinophora gossypiella* (Saunders), beet armyworm, *Spodoptera exigua* (Hübner), corn earworm, *Helicoverpa zea* (Boddie), gypsy moth, *Lymantria dispar* (L.), house cricket, *Acheta domesticus* (L.), and various beetles (Coleoptera) (Lindegren et al., 1979; Blinova and Ivanova, 1987; Cabanillas and Raulston, 1994; Grewal et al., 1999; Elawad et al., 2001).

Nematode yield is generally proportional to insect host size (Blinova and Ivanova, 1987; Flanders et al., 1996). However, IJ yield per mg insect (within host species), and susceptibility to infection, is usually inversely proportional to host size or age (Blinova and Ivanova, 1987; Shapiro et al., 1999; Dolinski et al., 2007; Dias et al., 2008). In addition to yield, ease of culture and susceptibility to IJs are important factors when choosing a host (Blinova and Ivanova, 1987; Shapiro-Ilan and Gaugler, 2002). Ultimately, the choice of host species and nematode for in vivo production should rest on nematode yield per cost of insect biomass, and the suitability of the nematode for the target pest (Blinova and Ivanova, 1987; Shapiro-Ilan et al., 2002a). Comparisons of cost efficiencies among different host species have rarely been studied. Blinova and Ivanova (1987) reported *T. molitor* to be superior in cost efficiency compared with *G. mellonella* and *Trichoplusia ni* for producing *S. carpocapsae*. In another study, Shapiro-Ilan et al. (2002a) observed that *H. bacteriophora* production in *T. molitor* was slightly advantageous relative to production in *G. mellonella*, whereas production efficiency for *S. carpocapsae* appeared to be equal in the two hosts (yield was higher in *G. mellonella*, but *T. molitor* were less expensive to produce). Finally, in addition to cost efficiency, nematode quality may also need to be considered when choosing a host because nematodes reared on various hosts may differ in quality (Abu

Hatab et al., 1998), and nematodes can become adapted to the host they are reared on (Stuart and Gaugler, 1996), which can have negative implications for biocontrol efficacy.

Inoculation parameters including dosage, host density, and inoculation method can have a profound effect on the efficiency of production. For each particular nematode and host species, optimization of host density and inoculation rate is recommended for achieving maximum yields (Shapiro-Ilan et al., 2002a). In general, the number of hosts exhibiting patent signs of nematode infection increases with nematode concentration and decreases with host density per unit area (Shapiro-Ilan et al., 2002a). A dosage that is too low results in low host mortality. In contrast, a dosage that is too high has been suggested to potentially result in failed infections due to competition with secondary invaders (Woodring and Kaya, 1988). Thus, intermediate dosages can be used to maximize yield (Boff et al., 2000). For example, rates of approximately 25−200 IJs per insect are usually sufficient (depending on nematode species and method of inoculation) for infecting *G. mellonella*, whereas higher rates are generally needed to infect *T. molitor* (e.g., 100−600 Ijs/insect). Crowding of hosts can lead to oxygen deprivation or buildup of ammonia, which suppresses nematode yield (Shapiro et al., 2000; Shapiro-Ilan et al., 2002a).

The inoculation method can also affect efficiency and yield. In vivo inoculation can be accomplished by pipetting or spraying nematodes onto a substrate, immersion of insects in a nematode suspension, or applying the nematodes to the insect's food. Immersion of hosts is generally more time-efficient but requires more nematodes than other procedures; for example, Shapiro-Ilan et al. (2002a) observed immersion of *G. mellonella* to be approximately four times more efficient in time than pipetting inoculum onto the hosts. However, some insects are more sensitive to being immersed than others. Relative to other methods, Blinova and Ivanova (1987) reported that infection of *T. molitor* by *S. carpocapsae* was increased using a feeding method. However, the feeding approach requires the additional step of removing infected cadavers from food remnants. Additionally, the food may introduce contaminants to the system. Therefore, inoculation procedures must be included in a cost-efficiency analysis before deciding on a method.

Several environmental factors including optimum temperature, adequate aeration, and moisture can affect yield (Burman and Pye, 1980; Woodring and Kaya, 1988; Friedman, 1990; Grewal et al., 1994; Shapiro-Ilan et al., 2002a; Dolinski et al., 2007). Temperature is critical during the rearing process as it affects both yield and lifecycle duration (time to emergence) (Grewal et al., 1994). Grewal et al. (1994) determined the optimum rearing temperature in *G. mellonella* for 12 species and strains of EPNs; optimum temperatures varied from 18°C to 28°C. For example, optimum rearing temperatures for certain strains of *S. feltiae* (a cold-tolerant species), *S. riobrave* (a heat-tolerant species), and *S. carpocapsae* (intermediate) were 18.5°C, 28°C, and 25°C, respectively. In addition to appropriate temperatures, adequate aeration (Burman and Pye, 1980; Friedman, 1990) and high levels of humidity are important environmental factors that must be maintained throughout the production cycle (Woodring and Kaya, 1988). An optimum balance between aeration (to avoid build-up ammonia or other harmful gases) and humidity should be reached, for example, using HEPA filtered air and a humidifying system.

10.2.3 Recent advances and future directions

As indicated above, the efficiency of in vivo production is limited by the costs of insects and labor. Therefore, the economics of in vivo production can be improved substantially by producing the insect hosts "in-house" and mechanizing the process (thereby reducing labor). Various steps in the in vivo production can be mechanized (inoculation, harvest, concentration). Indeed, mechanization of the entire process from insect production through harvest and packaging has been proposed (Morales-Ramos et al., 2012; Shapiro-Ilan et al., 2012c, 2016).

Several approaches to the mechanization of nematode inoculation and harvest have been developed or proposed. For example, improved inoculation devices have been developed to allow for mass inoculation on multiple shelves (Carr and Kolodny-Hirsch, 1993; Shapiro-Ilan et al., 2009b, 2016). A mechanized harvest device, LOTEK, allows for automated collection of IJs from stacked trays; the IJs are pumped to a central collection tank; unlike the White trap approach, harvesting does not require nematode migration to a water reservoir (Gaugler et al., 2002).

Shapiro-Ilan et al. (2011) introduced a single tray design where infected host cadavers are placed on a substrate intended as the formulation carrier (e.g., a gel) within the final package; once IJs emerge the cadavers are removed and the final nematode product is ready for shipment or storage. In this approach, the process is streamlined substantially because nematodes emerge directly into their formulation and package and thus additional concentration and packaging steps are removed from the process. The approach is also advantageous because it preserves host factors (e.g., ascaroside pheromones) from the host cadaver, bacteria and or nematodes that enhance EPN dispersal, and infectivity; indeed, these pheromones can be mixed IJs prior to field application to improve pest control performance (Wu et al., 2018; Oliveira-Hofman et al., 2019, Shapiro-Ilan et al., 2019).

Advances have also been made in improving insect host production for in vivo EPN production. The host's reproductive capacity can be enhanced by optimizing host density and lifecycle aspects of the rearing process (Morales-Ramos et al., 2012). Furthermore, Morales-Ramos et al. (2011a) developed a mechanized sifter to automatically separate insect hosts (*T. molitor*) of different sizes (thereby facilitating selection for nematode inoculation, continued culturing, etc.). Optimization of insect diets can also lead to improved efficiency in insect host production (Morales-Ramos et al., 2011b). Morales-Ramos et al. (2011b) used a self-selection regime to identify optimum ratios of carbohydrates (bran and potato) in the diet of *T. molitor*; recently optimum levels of lipid and protein supplements were also determined using the same technique (Morales-Ramos et al., 2014). Based on tri-trophic interactions, the development of the host diet can also be used to improve the quality and production of nematodes, for example, a program has been initiated to simultaneously improve the diet of *T. molitor* (including carbohydrate, protein and lipid content) for optimal host production and nematode fitness (Shapiro-Ilan et al., 2008a, 2012c).

An alternate approach to in vivo production is culture and delivery of EPNs in their infected host cadavers (Jansson et al., 1993; Shapiro and Glazer, 1996; Del Valle et al., 2008). In this approach, nematodes are applied to the target site in their infected hosts, and pest suppression is subsequently achieved by progeny IJs that emerge from the cadavers. Production and application of nematodes in infected hosts may be more efficient than other in vivo production methods because several labor-intensive steps are removed from the process, that is, harvest and concentration. Furthermore, laboratory studies have indicated that nematodes applied using the cadaver approach exhibited superior dispersal (Shapiro and Glazer, 1996), infectivity (Shapiro and Lewis, 1999), survival (Perez et al., 2003) and environmental tolerance (Gulzar et al., 2020) compared with nematodes applied using the standard approach in aqueous suspension. Pest control efficacy when using the cadaver application approach was also reported to be superior to standard application in aqueous suspension (Shapiro-Ilan et al., 2003a). The cadaver application approach has shown high levels of efficacy in the suppression of various pests including the black vine weevil, *Otiorhynchus sulcatus* (F.), citrus weevil, *Diaprepes abbreviatus* (L.), guava weevil, *Conotrachelus psidii* Marshall, large pine weevil, *Hylobius abietis* (L.), and small hive beetle, *Aethina tumida* (Murray) (Shapiro-Ilan et al., 2003a, 2010; Dillon et al., 2007; Del Valle et al., 2008).

One potential drawback to applying EPNs in infected hosts is that the cadavers may be fragile or stick together, particularly when a soft-bodied insect host is used such as *G. mellonella*. However, the application of cadavers may be facilitated through formulations that have been developed to protect cadavers from rupture and improve ease of handling, such as a starch matrix with clay coating or an encapsulation process (Shapiro-Ilan et al., 2001, 2010; Del Valle et al., 2009). Alternatively, rupture and sticking together can be avoided by using a hard-bodied insect as the host, such as *T. molitor* (Shapiro-Ilan et al., 2008b). The use of hard-bodied insects can be further enhanced using a mechanized device that automatically wraps the cadavers between two pieces of masking tape; the IJs emerge between two rolls of tape without any loss in yield (Morales-Ramos et al., 2010; Shapiro-Ilan et al., 2010). Another barrier to the cadaver application method is devising procedures to distribute the infected hosts on a large scale; to this end, mechanized equipment for field distribution has been developed (Zhu et al., 2011). In yet another novel approach to using EPN infected hosts, recently, nematodes applied in host cadavers were effective and highly persistent when added to bags of potting media for subsequent distribution to target pest sites (Deol et al., 2011).

10.3 In vitro production—solid culture

10.3.1 Basic method

The first attempt at EPN in vitro production was carried out on a solid medium axenically, but efforts to continuously produce EPNs failed because the required presence of the nematode's symbiotic bacterium was not yet known (Glaser, 1931). However, much later when the bacterial symbionts were discovered and their importance for EPN reproduction was recognized, a foundation was laid down for in vitro mass production of EPNs (Poinar and Thomas, 1966). Today, in vitro production of EPNs is based on introducing nematodes to a pure culture of their symbiont in a nutritive medium.

A prerequisite to in vitro production (using solid or liquid media) is the establishment of a monoxenic culture. Steps toward creating monoxenic cultures include isolation of the symbiotic bacteria and establishment of bacteria-free nematodes. The symbiotic bacteria can easily be isolated from nematode-infected insect larvae or surface-sterilized IJs. The bacteria can occur in two-phase variants, primary and secondary, which differ in several characteristics including dye absorption, response to biochemical tests, antibiotic production, and bioluminescence (for *Photorhabdus* bacteria). Although EPNs have been reported to grow on secondary variant symbionts, the primary variant is most conducive to growth and IJs tend to only retain the primary variant (Han and Ehlers, 2001). Single-phase I colonies can be selected

on indicator plates of nutrient bromothymol blue agar (NBTA) (per 1 L distilled water: peptone 5.0 g, beef extract 3.0 g, agar 15 g, bromothymol blue 0.025 g and 2,3,5-triphenyl tetrazolium chloride 0.04 g) and MacConkey (per 1 L distilled water: peptone 17 g, proteose peptone 3 g, lactose 10 g, bile salts 1.5 g, sodium chloride 5 g, neutral red 0.03 g, agar 13.5 g) (Akhurst, 1980) and cultured in lysogeny broth (per liter distilled water: NaCl 5 g, tryptone 10 g, yeast Extract 5 g) or yeast salts (per liter distilled water: NH4H2PO4 0.5 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl, yeast extract 5.0 g) broth. Stock cultures of bacteria are mixed with glycerol at 15% (v/v), and aliquots are frozen at −80°C.

More laborious is the establishment of axenic nematodes (for subsequent introduction to the pure bacteria culture and initiation of production). In earlier work, to create monoxenic cultures, surface-sterilized nematodes were added to a lawn of the bacterial symbionts (Akhurst, 1980; Wouts, 1981). However, Lunau et al. (1993) suggested that surface sterilization of IJs is insufficient to establish monxenicity because contaminating bacteria survive beneath the nematode's cuticle. Thus, an improved method was developed where axenic nematode eggs are obtained by rupturing gravid nematode females in an alkaline solution (Lunau et al., 1993; Han and Ehlers, 1998). Specifically, in this method, fertile nematode eggs from gravid females (*Steinernema* spp.) or hermaphrodites (*Heterorhabditis* spp.) are collected in Ringer's solution (per 1 L distilled water: NaCl 9.0 g, KCl 0.42 g, $CaCl_2$ 0.48 g, $NaHCO_3$ 0.2 g) and surface-sterilized in a solution of 2.5 mL 4 M NaOH, 0.5 mL 12% NaOCl, and 21.5 mL distilled water. After rinsing twice in sterile Ringer's solution, the eggs are transferred to sterile liquid media in which the J1 stage hatches. After 2 days, (as long as the medium is without growth of contaminants) the J1 are combined with their symbiotic bacteria growing on the fortified lipid agar (1.6% nutrient broth, 1.2% agar and 1% corn oil; Wouts, 1981). Once the monoxenic cultures are established, the nematode culture can be scaled-up in the solid phase or liquid phase.

The in vitro solid technique involves the following 4 steps: preparation of solid medium, inoculation with bacteria, inoculation with nematodes and harvest. The solid culture was first accomplished in two-dimensional arenas, for example, Petri dishes, using various agar media such as those based on dog food, pork kidney, cattle blood, and other animal products (House et al., 1965; Hara et al., 1981; Wouts, 1981). Wouts (1981) developed an improved medium (less expensive and more consistent from batch to batch) that included yeast extract, nutrient broth, vegetable oil, and soy flour. Subsequently, in vitro solid culture was advanced considerably by the invention of a three-dimensional rearing system involving nematode culture on crumbled polyether polyurethane foam (Bedding, 1981). This was a major breakthrough and opened the door for economic feasibility in commercial mass production. Several companies (Biotech Australia, De Groene Vlieg and Koppert Biological Systems, both from the Netherlands, and Bionema, Sweden, and a company named Hongrun from China) started to produce and sell EPNs for soil pests, mainly in greenhouses (Ehlers, 2001a). In the 3-dimensional solid media method, a liquid medium is mixed with foam, autoclaved, and then inoculated with bacteria followed by the nematodes. Nematodes are then harvested within 2−5 weeks (Bedding, 1981; Bedding, 1984) by placing the foam onto sieves immersed in water or onto sieve bags washed in a washing machine. Media for this approach was initially based on animal products such as pork kidney or chicken offal, but these media were later improved and may include various ingredients including peptone, yeast extract, eggs, soy flour, wheat flour, and lard (Han et al., 1992, 1993). The approach was later expanded to autoclavable bags with filtered air being pumped in (Bedding, 1984) and culture vessels comprising a metal tray with side walls and overlapping lids that allow gas exchange through a layer of foam. Large scale production was advanced further through various mechanisms including using bags with gas permeable Tyvac strips for ventilation, automated mixing and autoclaving, simultaneous inoculation of nematodes and bacteria, sterile room technology, and automated harvest through centrifugation (Gaugler and Han, 2002).

10.3.2 Factors affecting efficiency

The impact of nematode inoculum rate (IJs per unit of media) on yield varies among nematode strains (Han et al. 1992, 1993, Wang and Bedding 1998). For example, *S. carpocapsae* (Agriotos strain) produced optimum yields at an intermediate inoculum size (2000 IJs per g medium) (Han et al., 1993), whereas *S. carpocapsae* (CB2B strain) and *H. bacteriophora* (H06) were not affected by inoculum size (Han et al., 1992). Bacterial inoculum size does not appear to be important in yield determination (Han et al., 1992, 1993).

Temperature plays a crucial role in nematode development in solid culture (Han et al., 1992, 1993). A temperature of 27°C supported optimal production for *Steinernema* sp. CB2B and 25°C for *H. bacteriophora* H06 (Han et al., 1992). However, some species require lower or higher temperatures for growth and reproduction. Dunphy and Webster (1989) reported the optimum temperature of 30°C for *H. bacteriophora* (=*heliothidis*) on lipid agar. When cultured in soy flour medium, maximum yields of Pakistani strains of *S. pakistanense* Shanina, Anis, Reid Rowe & Maqbool, *S. asiaticum* Anis, Shahina, Reid & Rowe, *S. abbasi* Elawad, Ahmad & Reid, *S. siamkayai* Stock, Somsook & Kaya, *S.*

carpocapsae, H. indica and *H. bacteriophora* were obtained at 32°C ± 2°C, whereas *S. feltiae* produced the highest yields at 20°C ± 2°C (Salma and Shahina, 2012). Thus, temperature adaptation of nematodes for maximum production in solid culture is related to the specific species or strains.

Culture time, being inversely related to temperature should be optimized for maximum yield according to the needs of each nematode species or strain (Dunphy and Webster, 1989; Han et al., 1992, 1993). Increasing inoculum size can increase nematode growth and decrease culture time (Han et al., 1992). Longer culture times can provide higher yields yet nematode mortality may also increase with time (Han et al., 1992, 1993) and culture time must be weighed against the cost of space and diminishing returns.

Media composition is a major factor that affects yield in solid culture. Increasing the lipid quantity and quality leads to increases in nematode yield (Dunphy and Webster, 1989; Han et al., 1992). The most suitable array of lipid components reflects the nematode's natural host composition (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 2001). Nematode yields were significantly affected by the addition of water, yeast extract, and egg yolk for *S. carpocapsae* and by the addition of water and lard for *H. bacteriophora*, which indicated the importance of selecting suitable medium components and combinations for these nematode species (Han et al., 1995). Soy flour is one of the media components both in solid culture and liquid culture (Ehlers and Shapiro-Ilan, 2005). Media containing soya flour yielded the highest population compared to media without soy flour in in vitro solid production of eight Pakistani strains (Salma and Shahina, 2012); the species tested included *Steinernema pakistanense* Shahina, Anis, Reid, Rowe & Maqbool, *S. asiaticum*, *S. abbasi*, *S. siamkayai, S. carpocapsae*, *S. feltiae, Heterorhabditis indica* and *H. bacteriophora*. Other media ingredients that may have an effect on nematode yield may include salts and proteins (Dunphy and Webster, 1989).

Yields in solid culture also depend upon the innate reproductive capacities of different nematode species and strains. For example, *H. bacteriophora* strain HbNJ yielded an average of 1.4×10^9/bag (Gaugler and Han, 2002), while an *S. scapterisci* strain yielded an average of 4.8×10^8/bag (Bonifassi et al., 1999). On the same culture media and under the same culture conditions, *H. indica* strain LN2 yielded much more than *H. bacteriophora* strain H06 (9.3×10^5 IJs/g medium vs 5.1×10^5 IJs/g medium) (Han and Qiu, unpublished data).

10.3.3 Recent advances and future directions

Recently, advances have been made in expanding in vitro solid production of EPNs. For example, in China, a pilot factory was established for solid production of several EPN species based on the lower labor cost available in that country, and an improved media and mechanization process. The factors influencing production efficiency were explored and optimized, including medium development, the culture parameters, recovery of the IJ inocula induced by specific signal compounds, formation of the IJs, extraction and harvest (Han et al., 1995, 1997). A company, under the guidance of the Institute of Zoology, Guangdong Academy of Sciences, is currently in commercial production; products from the solid culture system include *S. carpocapsae*, *S. feltiae*, *S. longicaudum, H. bacteriophora*, and *H. indica*, which are provided for field trials in China and for internal and international markets.

10.4 In vitro production—liquid culture

10.4.1 Basic method

EPN in vitro liquid culture was first attempted by Stoll (1952) using raw liver extract in shaken flasks, and was developed further by several researchers (Ehlers, 2001b; Shapiro-Ilan et al., 2012b). It is a complex rearing process which demands medium development and optimization, understanding of the biology of the nematode and their bacteria, and bioreactor development including understanding and controlling the process parameters. Collaboration between researchers in the public and the private sector contributed largely to the establishment of in vitro liquid culture.

Successful implementation of the liquid culture process faces the opposing challenges of supplying enough oxygen while preventing excessive shearing of nematodes (Pace et al., 1986; Buecher and Popiel, 1989; Friedman et al., 1989; Friedman, 1990). Initially, the issue was addressed using various approaches such as relying on bubbling, for example, with a downward sparger, coupled with limited agitation (Pace et al., 1986), or using an airlift fermenter coupled with a variable agitation regime (Friedman et al., 1989). Innovations in mixing and aeration have been subsequently introduced including internal (Strauch and Ehlers, 2000) and external (Neves et al., 2001) bioreactors. Internal loop vessels have baffles placed inside the single vessel, which create the channels required for circulation, whereas in external loop vessels circulation takes place through separate conduits. Another problem observed during agitation is foaming, which

can be reduced by changes in the bioreactor design (Strauch and Ehlers, 2000) and the use of antifoam or defoaming agents (Gaugler and Han, 2002).

The first attempt to use bioreactors was described by Pace et al. (1986) and the first commercial application of the liquid culture technology was made by the company Biosys (Palo Alto, California). The company was incorporated in 1987 and soon started to produce liquid culture nematode products. In 1992, large-scale production of *S. carpocapsae* began and was scaled-up to volumes of 80,000 L. Today commercial EPNs resulting from liquid culture are produced by several companies.

In liquid culture, symbiotic bacteria are generally first introduced followed by the nematodes (Buecher and Popiel, 1989; Surrey and Davies, 1996; Strauch and Ehlers, 2000; Johnigk et al., 2004; Shapiro-Ilan et al., 2012b). A variety of ingredients for liquid culture media have been reported including soy flour, yeast extract, canola oil, corn oil, thistle oil, egg yolk, casein peptone, milk powder, liver extract and cholesterol (Surrey and Davies, 1996; Ehlers et al., 2000; Yoo et al., 2000). Media and species effects impact culture times in liquid culture, which may be as long as three weeks (Surrey and Davies, 1996; Chavarria-Hernandez and de la Torre, 2001) though many species can reach maximum IJ production in two weeks or less (Friedman, 1990; Ehlers et al., 2000; Strauch and Ehlers, 2000; Yoo et al., 2000; Neves et al., 2001).

EPN cultures in liquid are particularly vulnerable to contamination due to the even distribution of the fluids and organisms obtained through the mixing of liquid in bioreactors and based on the long process time, (Ehlers and Shapiro-Ilan, 2005). A liquid culture process starts with the inoculation of a pure culture of the symbiotic bacteria in its exponential growth phase into a suitable sterilized liquid medium. The main considerations in scaling up production are to ensure a good quality of bacteria to trigger nematode development, to provide a suitable food source, and to synchronize nematode development in order to obtain a pure IJ suspension at the end of the process (Peters et al., 2017). The symbiotic bacteria of both genera (*Xenorhabdus* and *Photorhabdus*) may switch between different morphologically and metabolically distinct phases (Akhurst, 1980; Boemare et al., 1997), some of which were shown to produce inferior yields in liquid culture processes of *Heterorhabditis* (Han and Ehlers, 2001) and *Steinernema* (Hirao and Ehlers, 2009). Secondary phase bacteria result in a lower proportion of IJs developing than primary form bacteria (Hirao and Ehlers, 2009). The phase variation is by definition not correlated to any changes in bacterial DNA (Owuama, 2001; Park et al., 2007). Phase variation was shown to be induced by osmotic stress, heat treatment or by culturing bacteria at low oxygen levels (Krasomil-Osterfeld, 1994, 1995). To avoid phase variation, bacteria should be cultured in the same media and oxygen stress should be avoided in any scaling-up step. The bacteria should then be grown to the late stationary phase before nematodes are added (Johnigk et al., 2004). High bacteria concentrations increased recovery and final yield in *S. carpocapsae* and *S. feltiae* (Hirao and Ehlers, 2009).

Moreover, nematode yields can be severely reduced by the presence of nonsymbiotic microbes; such contamination can prevent further scale-up. However, good bacterial growth (primary phase bacteria) at the beginning of the process produces antibiotics that inhibit contamination during the 3 weeks nematode production process. The monoxenicity of the cultures must be ensured and verified from the onset of inoculum production. Monoxenic cultures to be used as inocula can be stored on shakers at 20 rpm and low temperature (generally 4°C, but for some nematode species it should be higher) for several months until they are introduced into the bioreactor. Strain collections of nematodes can be kept in liquid nitrogen (Popiel and Vasquez, 1991). Cultures are always preincubated for 24−36 h with the specific symbiont bacteria before IJs are inoculated. The inoculum density for the symbiotic bacteria is between 0.1% and 1% of the culture volume. The inoculum of the nematodes varies depending on species and media composition. An optimal number of adults per mL can be calculated, which is defined by the percentage of IJs likely to recover. The recovery of *S. feltiae* and *S. carpocapsae* decreased at lower bacterial densities (Hirao and Ehlers, 2009). If the concentration of adult nematodes feeding on the bacteria is too low, a larger proportion of the F1 generation will develop into adults and start a second-generation (Peters et al., 2017). If the proportion is too high, there will not be enough bacteria left to support the formation of progeny. Generally, the nematode inoculum is between 5% and 10% of the culture volume. The culture medium should have a pH between 5.5 and 7.0 when the culture is started. The pH appears to be well regulated by the organisms themselves. The pH is not controlled but monitored to identify switches in the metabolic activity of the symbiotic bacteria (Peters et al., 2017). Oxygen supply must be maintained at approximately 30% saturation, which may help to prevent the bacteria from shifting to the secondary phase. The aeration rate (L/min of sterile air supplied to the liquid) and the stirrer speed are used to control dissolved oxygen (DO). Increasing the aeration and stirring/agitation rates often increase foaming. The addition of agents such as silicon oil usually prevents foaming. However, it should be used carefully, because higher concentrations can be detrimental to the nematodes. Compared to the pure bacteria culture, the maximum stirrer speed is lowered after nematodes are added to avoid shear stress, which may kill the fragile hermaphrodites (Peters et al., 2017). The increased oxygen uptake rate can therefore not be adequately compensated by

increasing the stirrer speed and the DO% drops below the set point, which does not, however, affect the nematode vitality. On the other hand, for the production of inoculum inside Erlenmayer flasks, the nematode is not affected by the increasing the speed of rotation, being in fact favored by its increase up to 280 rpm due to the increase of aeration in the liquid medium (Leite et al., 2016a,b,c).

Owing to the potential of *Xenorhabdus* and *Photorhabdus* spp. to metabolize almost every kind of protein-rich medium, the selection of appropriate culture media for EPN production can largely follow economic aspects (Ehlers and Shapiro-Ilan, 2005). A standard medium should contain a carbon source (like glucose or glycerol), a variety of proteins of animal and plant origin, yeast extract and lipids of animal or plant origin (Pace et al., 1986; Friedman et al., 1989; Han et al., 1993; Surrey and Davies, 1996; Ehlers et al., 1998). The osmotic strength of the medium must not surpass 600 millimol/kg. Improvements in the medium and adaptation to the requirement of different species are feasible approaches to increasing yields (Ehlers, 2001a). Essential amino acid requirements have only been defined for *S. glaseri* (Jackson, 1973). Nematodes have nutritional demands for sterols, but they can metabolize the necessary sterols from a variety of steroid sources (Ritter, 1988) that are provided through the addition of lipids of animal or plant origin. According to Leite et al. (2016a), peanut oil provided numerically the highest growth of *S. feltiae* among six other plant-based oil, while pork fat was detrimental to the nematode growth due to its solidification in the liquid medium at room temperature and consequently low solubility. For the nitrogen source experiments, yeast extract provided the lowest yields, while egg yolk + egg white increased nematode yield, especially when these two nitrogen sources were combined. Also, the addition of 2.5% glucose increased nematode yields.

In general, *S. carpocapsae* requires proteins of animal origin (Yang et al., 1997) and it is unable to reproduce without the addition of lipid sources to the medium, whereas *H. bacteriophora* produces offspring in a liquid medium without the addition of lipids (Han and Ehlers, 2001). *Photorhabdus luminescens* provides or metabolizes essential lipids; however, lipids should always be added to increase the total IJ fat content. The lipid composition of the medium has an effect on the fatty acid composition of the bacteria and IJs (Abu Hatab et al., 1998), low fatty content of IJ can reduce efficacy (Patel et al., 1997a,b).

Another important issue for nematode production in liquid culture is the viscosity of the medium. The medium viscosity increases naturally along the nematode production process (Chavarría-Hernández et al., 2010), but a minimum viscosity may help the starting growth in the liquid culture, especially for the nematode recovering after its inoculation as IJs. Leite et al. (2016c) found the higher recovering rate of *S. feltiae* IJs by increasing the viscosity of the medium with the addition of 0,2% agar. The addition of 0.2% agar to the liquid medium and increasing the aeration rate by using larger flasks with higher agitation speed (280 rpm) increased bacteria growth, nematode recovery and final yield. Media containing agar (0.4% and 0.6%) increased nematode yields only when cultures were grown at low agitation speed (180 rpm). These three conditions (agar, larger flask and higher agitation speed) favored the growth of bacteria and nematodes by improving oil distribution and dissolved oxygen in the medium.

10.4.2 Factors affecting efficiency

Although both steinernematids and heterorhabditids share the requirements of adequate aeration (without shearing), the approaches for maximizing the yield of the two genera in liquid culture differ due to their life cycles and reproductive biology. Given that steinernematids (except one species) occur only as males and females and are capable of mating in liquid culture (Strauch et al., 1994), maximization of mating is paramount and can be achieved through bioreactor design and regulation of aeration (Neves et al., 2001). However, maximization of mating is not applicable for heterorhabditid production in liquid culture because the first generation is exclusively hermaphroditic and, although subsequent generations contain amphimictic forms, they cannot mate in liquid culture (Strauch et al., 1994). Thus, maximizing heterorhabditids yields in liquid culture depends on the degree of recovery (the developmental step when IJs molts to initiate completion of their life cycle).

Whereas levels of heterorhabditids recovery in vivo tend to be 100%, recovery in liquid culture can range from 0% to 85% (Ehlers et al., 2000; Jessen et al., 2000; Yoo et al., 2000). Recovery may be affected by various factors including nutrition, aeration, CO_2, lipid content, and temperature (Strauch and Ehlers, 2000; Ehlers et al., 2000; Jessen et al., 2000; Yoo et al., 2000). Recovery can be influenced during the bacterial preculture phase, prior to nematode introduction. A higher bacterial density leads to a higher food signal concentration, which in turn produces a higher recovery rate (Hirao and Ehlers, 2009). Nematodes should therefore be inoculated during the late stationary growth phase (Johnigk et al., 2004). The period starts with the increase of the pH after its distinct minimum at $pH < 8.0$ (Johnigk et al., 2004). Glucose fed-batch reactors can increase bacterial density (Jeffke et al., 2000) and enhance food signal production, and thus may be used to increase IJ recovery (Ehlers and Shapiro-Ilan, 2005). Jessen et al. (2000) reported that

increasing the CO_2 concentration in the medium enhanced IJ recovery. However, the influence of decreasing pH caused by the CO_2 concentration was excluded. A pH below 6.5 significantly reduced the IJ recovery. A protein source like soybean can improve symbiotic bacterial growth and nematode recovery, which thereby promotes nematode production (Cho et al., 2011). Recovery in secondary phase bacteria is lower than in primary phase bacteria (Han and Ehlers, 2001; Hirao and Ehlers, 2009). No significant influence of inoculum density on IJ recovery of *S. carpocapsae* and *S. feltiae* has been detected; but a significant correlation between recovery and the age of IJ inoculum was detected for *S. carpocapsae* (Hirao and Ehlers, 2010). According to Leite et al. (2016a), advancing the inoculum age from 7 to 28 days after the starting of nematode growth in the flasks, resulted increasing in nematode yield in the liquid fermentation process (maximum of 180,000 IJs/mL) but not in the solid-state process (maximum of 120,000 IJs/mL). A possible reason is that in liquid culture the nematode is free to reproduce and multiply, while in solid-state its reproduction and multiplication might be limited by the pores of the sponge.

Yield from liquid culture may also be affected by other factors including media, inoculum parameters, nematode species, culture temperature and culture time (Han, 1996; Ehlers et al., 2000). The central component of the liquid culture media is the lipid source and quantity (Abu Hatab et al., 1998; Yoo et al., 2000). Other nutrients that have been reported to affect yield positively include the glucose content (Jeffke et al., 2000), yeast extract content (Chavarria-Hernandez and de la Torre, 2001) and soybean protein (Cho et al., 2011). Similar to the other production approaches, nematode yield in liquid culture is generally inversely proportional to the size of the species (Ehlers et al., 2000). Some of the maximum average yields reported include 300,000 and 320,000 IJs/mL for *H. bacteriophora* and *S. carpocapsae*, respectively (Han, 1996), 138,000/mL for *H. megidis* (Strauch and Ehlers, 2000), 200,000 Ijs/mL for *S. feltiae* (Leite et al., 2016a,b,c) and 450,000 IJs/mL for *H. indica* (Ehlers et al., 2000).

The temperature has an influence on the time scale of nematode development. For example, Ehlers et al. (2000) noted that recovery is influenced by culture temperature. The optimal culture temperature varies from species to species, for instance, the optimal culture temperature is 25°C for *S. carpocapsae* and *S. feltiae*. The equipment and type of bioreactors used will also impact yield. For example, although flat-blade impellers, bubble columns, airlift and internal loop bioreactors, have all been successfully tested (Pace et al., 1986; Surrey and Davies, 1996; Ehlers et al., 1998), a direct comparison with flat-blade impeller-stirred tanks or air-lift bioreactors internal loop bioreactors always yielded higher IJ concentrations (Ehlers and Shapiro-Ilan, 2005).

10.4.3 Recent advances and future directions

Some recent advancements in liquid culture technology serve to increase the quality and efficiency of production through various processes such as optimizing media and bioprocess kinetics through modeling (Chavarria-Hernandez et al., 2006, 2010), as well as improvements in the inoculum and bacterial cell density (Hirao and Ehlers, 2010), the timing of inoculation (Johnigk et al., 2004), improving beneficial traits (such as heat and desiccation tolerance) in *Heterorhabditis* spp. (Mukuka et al., 2010; Anbesse et al., 2012), and downstream processing (Young et al., 2002). Future research and development in liquid culture, focusing on media optimization and bioreactor design, are expected to lead to additional benefits such as higher yields and reduced costs.

10.5 Analysis and conclusion

10.5.1 Comparison of production methods

A summary of the advantages and disadvantages of each EPN production approach is illustrated in Table 10.2. Issues of concern, include capital outlay, technical expertise, nematode quality, labor, and cost-efficiency. In assessing the merits of the approaches among these issues, in vivo production and in vitro liquid culture are the extremes and in vitro solid culture is intermediate between them. For example, the level of capital outlay and expertise required is lowest for in vivo production and highest for in vitro liquid production, and intermediate for in vitro solid production (Friedman, 1990; Gaugler and Han, 2002; Shapiro-Ilan et al., 2012b). Another advantage to in vivo production is the ease of adapting the process to new/different nematode species; generally, the process can remain the same except for some slight modifications (such as temperature regimes). In contrast, in vitro techniques may require substantial modification when adapting to new nematode species based on media requirements, optimization of fermentation parameters and downstream processing. Based on the versatility, and low levels of technology and expertise required, in vivo production may be most appropriate for small startup companies or in lesser developed countries or regions (where labor may be less expensive).

TABLE 10.2 A comparison of production approaches for entomopathogenic nematode.

	Production approach		
Issue	**In vivo**	**In vitro—solid**	**In vitro—liquid**
Capital outlay	Low	Intermediate	High
Required Expertise	Nominal	Intermediate	Extensive
Ease of achieving quality	Easy	Difficult	Difficult
Labor required	High	Intermediate	Low
Economy of Scale	Low	Intermediate	High
Ease of adaptation to new nematode species	Easy	Difficult	Difficult

On the other hand, a major disadvantage of in vivo production is the labor costs, which are the highest for in vivo production and lowest for in vitro liquid production (in vitro solid production again being in between). Heretofore, due to the high labor costs, space requirements, and cost of insects, the economy of scale (cost efficiency) tends to be the lowest for in vivo production and the highest for liquid production. Thus, on a global scale, the vast majority of nematode production is by far currently accomplished using in vitro liquid production.

Despite the reduced economy of scale relative to other production methods, in vivo production has managed to sustain itself as a cottage industry throughout the evolution of commercial in vitro enterprises (Shapiro-Ilan and Gaugler, 2002). In fact, in some markets, in vivo produced nematodes have remained competitive with, or even outcompeted, in vitro produced nematodes due to weakened consumer or distributor confidence in the quality of the in vitro product. Possibly, in vivo production will continue to expand based upon advancements in mechanization as described above.

In vitro solid production has several advantages. It does not require high technology inputs and large investments. The effect of phase variation on the yields is less than in liquid culture (Han and Ehlers, 2001). When it comes to large-scale production, the disadvantages can be overwhelming. In vitro solid production is labor-intensive, vulnerable to contamination during up- and downstream processing and is difficult to monitor online. The uneven distribution of the nematodes in the medium prevents systematic sampling and thus improvement of the technique is required. However, today, a few companies still produce EPNs in solid culture, for instance, Bionema (http://www.bionema.se), Andermatt Biocontrol AG (http://www.biocontrol.ch), BioLogic USA (http://www.bio-logic.us) and Guangzhou Greenfine-Biotechnology Co. Ltd (China). Recently, the free-living nematode, *Panagrellus redivivus* (L.), a promising food source for first-feeding fish and crustaceans, was successfully cultured on *Xenorhabdus* bacteria in a solid in vitro system (Cao et al., 2008). In developing countries, the in vitro solid culturing system is still superior to liquid culture technology (Ehlers et al., 2000). Similar to in vivo production, efficiency can be increased through mechanization (labor reduction) and media enhancement (Gaugler and Han, 2002; Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan et al., 2016).

An issue that has been in contention is the relative quality of nematodes produced using the various methods. Various authors and practitioners contend or have found evidence that in vitro produced nematodes can be inferior in quality compared with in vivo produced nematodes. For example, several studies indicate quality of nematodes produced using solid culture to be similar to nematodes produced by in vivo methods (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 1999; Gaugler and Georgis 1991), yet Yang et al. (1997) reported reduced fitness in *S. carpocapsae* produced in solid culture compared with in vivo culture. Furthermore, several reports also indicated reduced quality and field efficacy of in vitro liquid produced EPNs relative to those produced in vivo (Gaugler and Georgis, 1991; Cottrell et al., 2011), whereas others did not detect any differences (Gaugler and Georgis, 1991; Shapiro and McCoy, 2000a). In contrast, to our knowledge, there have been no reports in which in vitro produced nematodes caused superior field efficacy compared with in vivo produced nematodes.

The differences between production methods can vary by EPN species. For example, Gaugler and Georgis (1991) reported that the production method (in vivo, solid culture, or liquid culture) did not impact the efficacy of *S. carpocapsae* for control of the Japanese beetle, *Popillia japonica* Newman, whereas the efficacy of *H. bacteriophora* produced in liquid culture was inferior to *H. bacteriophora* produced using either of the other methods.

The quality of in vitro production can vary from batch to batch. For example, in one field trial, Cottrell et al. (2011) observed reduced field efficacy in the liquid produced *S. carpocapsae* relative to in vivo produced nematodes, yet there

was no difference in another trial (the nematodes were from the same commercial source, but trials were conducted using different batches). The quality of in vivo produced nematodes may also vary based on production sources (Gaugler et al., 2000). Therefore, it is conceivable that at least in some (if not many) cases, differences observed in nematode quality stem from batch variation rather than innate differences in production technique. Indeed, despite a few reports otherwise, there are vast numbers of examples where liquid produced EPNs have produced high levels of efficacy in a consistent manner. Additionally, with improvements in bioreactor design, media, and other parameters the quality of in vitro liquid produced nematode continues to advance (Chavarría-Hernández et al., 2010; Hirao and Ehlers, 2010).

10.5.2 Strain selection, improvement and stability

One aspect that will improve EPN production regardless of culture method is the use of superior nematode strains. Strains that provide higher yields will obviously increase cost-efficiency. Furthermore, strains that possess superior biocontrol traits (e.g., virulence, environmental tolerance, host-finding ability) will lead to improved cost-efficiency because treatment of the target site will require fewer nematodes per unit area.

Selecting the most suitable EPN strain from a variety of candidates can be addressed simply by screening existing species and strains that may possess superior levels of desired traits. Additionally, surveys can be implemented to discover new strains, which can then be screened in comparison to existing strains; such surveys have been conducted extensively for EPNs (Shapiro-Ilan et al., 2003b, Bruck, 2004; Campos-Herrera et al., 2008; Malan et al., 2011). The screening process is often accomplished by first narrowing down the number of candidates in laboratory comparisons. The laboratory-based comparisons should address reproductive capacity in the culture system, as well as test various desired traits including efficacy against desired target pests under simulated field conditions.

Once a candidate EPN strain is selected, the nematode's reproductive capacity should be tested under full-scale production conditions (rather than just pilot scale). Furthermore, verification of pest control efficacy in the field is critical and should be addressed using the nematode in its final formulated product. An entomopathogen that shows high virulence in the controlled laboratory environment could fail to suppress the target pest in the field due to various biotic or abiotic factors that may render the organism incompatible. For example, some laboratory strain selections that later proved successful in the field include *S. riobrave* (355 strain) and *H. indica* (HOM1 strain) for control of the citrus weevil, *D. abbreviatus* (Duncan and McCoy, 1996; Shapiro et al., 1999; Shapiro and McCoy, 2000b), and *S. riobrave* (355) for control of *C. nenuphar* (Shapiro-Ilan et al., 2002c, 2004; Pereault et al., 2009). In contrast, *S. feltiae* was highly virulent to *C. nenuphar* in the laboratory but failed to control the pest under field conditions in Georgia peach orchards (Shapiro-Ilan et al., 2004). Indeed, strain selection that focuses primarily on virulence or mass production and ignores habitat preferences of the pathogen has often been unsuccessful (Shapiro-Ilan et al., 2012a).

If existing or newly discovered entomopathogen strains are not sufficient to reach expected production yields, or cannot achieve desired levels of biological control efficacy, another option is to improve selected candidate strains through genetic approaches. Genetic improvement approaches can be directed toward the enhancement of single or various beneficial traits, such as reproductive capacity, suitability to production or formulation regimes, virulence, environmental tolerance, etc. Methods for improvement may include nonmolecular or molecular approaches. One nonmolecular method entails directed selection for desired traits. Selection for improved virulence can be obtained by passing an entomopathogen through a susceptible host (Steinhaus, 1949; Daoust and Roberts, 1982). Some examples of genetic selection for other traits in EPNs include improvements in host-finding (Gaugler et al., 1989) and nematicide resistance (Glazer et al., 1997). However, directed selection for a particular trait can inadvertently select for an inferior level of another trait (Gaugler, 1987). For example, a loss in storage capacity was observed in EPNs that had been selected for improved host finding (Gaugler et al., 1990).

Hybridization is another nonmolecular approach to strain improvement. In this approach, the transfer of beneficial traits from one strain to another is accomplished through controlled breeding. The use of hybridization was first demonstrated in EPNs by Shapiro et al. (1997); heat tolerance was transferred from one *H. bacteriophora* strain to another. Given that heterorhabditids produce both hermaphroditic and amphimictic forms, extra care must be taken to ensure that nematode progeny in controlled crosses arises from intended mating regimes rather than self-fertilization. Shapiro et al. (1997) accomplished this by using marker mutations. Hybridization in steinernematids is more straightforward in that only amphimictic forms exist. An example of hybridization in steinernematids is illustrated in the study of Shapiro-Ilan et al. (2005), which used hybridization of *S. carpocapsae* strains to develop superior environmental tolerance and virulence (to the pecan weevil), *Curculio caryae* (Horn) (Shapiro-Ilan et al., 2005). The two nonmolecular approaches (selection and hybridization) have also been combined for development of superior EPN strains (Mukuka et al., 2010).

Progress has been made toward molecular approaches for improving EPNs; these procedures entail direct genetic modification of the organisms. For example, a strain of *H. bacteriophora* was improved for heat tolerance via transformation using a heat shock protein originating from *Caenorhabditis elegans* (Maupas) (Gaugler, et al., 1997). A risk assessment study concluded that the transgenic organism was an unlikely environmental risk and thus the approach to improving biocontrol was considered viable (Gaugler, et al., 1997). The sequencing of entire genomes of EPNs and their symbionts (Duchaud et al., 2003; Bai and Grewal, 2007; Ciche, 2007; Bai et al., 2009; Schwartz et al., 2011) is expected to continue expanding and will undoubtedly enhance the potential for genetic strain improvement programs using molecular or nonmolecular approaches.

Once a desirable strain is chosen for mass production (based on existing cultures or stemming from an improvement program), it is imperative to ensure the stability of that strain. Regardless of the culture method, production efficiency and biocontrol efficacy can be jeopardized by attenuation of beneficial traits, which can result from repeated subculturing. This phenomenon of trait deterioration can be due to genetic factors (e.g., inbreeding, drift, inadvertent selection), or nongenetic factors (like disease or nutrition) (Tanada and Kaya, 1993; Hopper et al., 1993; Chaston et al., 2011). Trait deterioration has been observed during laboratory culturing of EPNs; relatively rapid loss of various traits was reported including virulence, environmental tolerance, reproductive capacity, and host-finding (Shapiro et al., 1996; Wang and Grewal, 2002; Bai et al., 2005; Bilgrami et al., 2006). Both the nematodes and their bacterial symbionts are subject to trait loss (Bilgrami et al., 2006; Wang et al., 2007), and the cause was reported to be (at least in part) genetically based with inbreeding depression being more prominent an issue than inadvertent selection (Bai et al., 2005; Adhikari et al., 2009; Chaston et al., 2011).

Therefore, manufacturers must implement precautions against strain deterioration. One approach to reduce trait deterioration is to maintain genetic diversity by storing isolates in liquid nitrogen (Nugent et al., 1996) or by minimizing subculturing (Roush 1990). Nematodes can be placed in liquid nitrogen for long-term storage; subculturing can be minimized by using the frozen material as seed cultures. There are, however, drawbacks to this approach. First, subculturing is inevitable because seed cultures eventually get used up and because mass culture or experiments routinely require many nematode generations, for instance, the number of generations needed for scale-up in liquid culture from shake flask to final bioreactor is extensive. Furthermore, because only small quantities of nematodes can be stored in each cryovial, and mortality during storage can be high (Nugent et al., 1996), founder effects may be pronounced when nematodes are stored in nitrogen. Indeed, Wang and Grewal (2002) observed a reduction in desiccation tolerance of *H. bacteriophora* during storage in liquid nitrogen. One option to overcome founder effects (at least to some extent) is to optimize cryopreservation techniques to increase the number of surviving IJs (Bai et al., 2004). Nonetheless, few EPN researchers or commercial producers use liquid nitrogen because strains vary in their adaptability to cryogenic storage (Nugent et al., 1996), it is expensive and factors like mechanical failure, human error, or neglect can result in complete loss of genetic material. Another approach is to supplement genetic diversity in the laboratory by re-collecting fresh nematode isolates from their source, or mixing strains. Collection of fresh material, however, takes time, may introduce disease and may be unreliable because it depends on the isolate remaining where it was last found. Mixing strains may reduce frequencies of desired alleles and, like the other methods, is only a temporary solution because subculturing is still inevitable.

Another approach to deterring trait deterioration in EPN strains that has recently been developed is the creation of homozygous inbred lines (Bai et al., 2005; Shapiro-Ilan et al., 2012b). Homozygous inbred lines in biocontrol agents were hypothesized to be impervious to certain genetic processes that impact trait stability (Hopper et al., 1993). The approach was first tested and validated in EPNs by Bai et al. (2005); selected inbred lines of *H. bacteriophora* remained stable during serial culture whereas their wild-type parent strains deteriorated in various traits (virulence, environmental tolerance and host-finding). Thus, Bai et al. (2005) proposed that manufacturers create numerous inbred lines from promising candidate strains, and select the lines that display high levels of desirable traits. Following further testing and validation of these selected lines (in full-scale production operations and biocontrol efficacy tests under field conditions) the inbred populations can then be used for commercial production. The approach provides strains that are both superior in production and biocontrol traits and stable during culture conditions. Indeed, the approach of combining novel strain discovery with the creation of inbred lines has been demonstrated to enhance pest control efficacy (Sharifi-Far et al., 2018).

In a variation of the inbred line approach developed by Bai et al. (2005), Anbesse et al. (2012) recommend the creation of multiple heterorhabditid inbred lines in liquid culture (within a single tank). Heterorhabditids cannot mate in liquid culture and therefore all progeny are produced by self-crossing (via hermaphrodites) (Ehlers and Shapiro-Ilan, 2005); this process automatically creates multiple inbred lines. Similar to the study of Bai et al. (2005), in which inbred lines were generated in vitro on agar plates, the inbred lines produced during liquid culture deter trait deterioration

(Anbesse et al., 2012). Anbesse et al. (2012) argue that the multiple inbred line approach (in liquid culture) is superior to the selection of only one or a few inbred lines (as in Bai et al., 2005) because genetic diversity is greater in the former. However, as long as a single (or hybridized) inbred line has been proven to have high biocontrol abilities (as is recommended prior to commercial production), genetic variation is not necessary; indeed, true-bred lines are used ubiquitously in crop production and animal husbandry. In fact, a drawback in allowing broad genetic diversity is that once the nematode population leaves the fermentation tank, the multitude of inbred lines in the nematode population (from that production batch) will be subject to trait deterioration in a similar manner as the wild type population. On the other hand, the likelihood that postapplication deterioration would occur is minimal because EPNs are usually applied in an inundative manner with little or no recycling expected (Shapiro-Ilan et al., 2012a), and thus both approaches (multiple inbred lines and single or hybrid lines) may be viable. One drawback to the liquid culture approach that cannot be circumvented is that it only works for heterorhabditids. The creation of inbred lines for steinernematids must be done outside of liquid culture to avoid free-mating such as via controlled sibling mating.

10.6 Conclusion

EPNs are commercially produced biocontrol agents that have become well established in several markets. The application of EPNs continues to increase on several continents. All three production approaches have played a role in the success of EPNs in the global marketplace, and all three will continue to contribute to EPNs expanded success in the future. In vitro liquid production is the most economical approach and is likely to continue to dominate the quantity of EPN production worldwide. Yet in some cases (where labor is less expensive) in vitro solid production may remain competitive. Although in vivo production is the least economical approach, it will likely continue to be appropriate for certain niche markets or for certain small or start-up companies; improvements to in vivo production may increase cost efficiency, yet it is unlikely the economy of scale will ever reach that of in vitro approaches.

Despite the success of EPNs in achieving commercial level control for a variety of pests, EPN products remain cost-prohibitive in many markets. Thus, additional advances to increase production efficiency and reduce costs are required. For in vitro production, further advances in fermentation approaches and parameters will lead to reduced costs. In vivo production and in vitro solid production will continue to benefit from streamlining of processes (reducing labor). Additionally, higher efficiency for in vivo production can be achieved through improvements in insect culture methods or the discovery of improved hosts for increased yields. Improvements in downstream processing (concentration and formulation) will also contribute substantially to increased cost efficiency for all production methods. As market demand for EPNs increases, additional scale-up of production will provide major benefits in reducing costs. Strain improvement programs may also lead to a reduction in EPN production or application costs. We recommend developing some improvement programs that are specifically focused on enhancing traits required for higher EPN production levels (e.g., reproductive capacity) as the majority of improvement endeavors thus far have focused on enhancing traits required in postproduction application. An alternative approach to EPN production that may contribute to market expansion would be developing grower-based or cooperative level "do it yourself" ventures (this could be accomplished using any of the three production methods); this concept has been discussed by a number of researchers but thus far has not been attempted in earnest.

To achieve expanded success in biocontrol, as production of EPNs increases, the application of the EPN products will need to fit into existing or emerging IPM strategies. Therefore, opportunities that facilitate incorporation into crop management systems should be seized, for instance, developing new (compatible) application methods or leveraging synergies between EPNs and other pest management tactics (Koppenhöfer and Grewal, 2005; Shapiro-Ilan et al., 2012a). As the use of broad-spectrum chemical insecticides decreases due to environmental and regulatory concerns, and progress in EPN production and application advances, we anticipate that the importance of EPNs as an integral component of sustainable pest management systems will evolve into a more prominent role.

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Chapter 11

Mass production of entomopathogenic fungi—state of the art*

Stefan T. Jaronski
Jaronski Mycological Consulting LLC, Blacksburg, VA, United States

11.1 Introduction

In 1835, Agostino Bassi demonstrated that a fungus could cause a deliberately transmissible disease in silkworms (Steinhaus, 1956; Lord, 2007). This discovery could be considered the birth of the potential to control insects with fungi. In the late 1870s, Metschnikoff observed a high proportion of *Metarhizium*-killed sugarbeet curculio *Cleonus punctiventris* Germar and proposed the concept of controlling sugarbeet curculio *C. punctiventris* Germar with conidia of a fungus, *Metarhizium anisopliae* (Metsch.) Sorokin *sensu lato* artificially produced on sterile brewer's mash (Metchnikoff, 1880; Steinhaus, 1956). His work was extended by Krassilstschik, who established a production facility using beer mash to produce a considerable amount of conidia for distribution (Krassilstschik, 1888). It was not until the 1970s and 1980s that interest in microbial agents increased when the adverse environmental impacts of chemical pesticides were better understood and alternatives began to be sought. During the last decades of the 20th Century commercialization efforts of fungi in the US were initially limited but efforts increased in several countries, most notably Brazil, Cuba, Czechoslovakia, the USSR, and China, more economic as well as environmental reasons.

The first two decades of the millennium have seen a proliferation of fungus-based products, "mycoinsecticides." Today, well over 200 commercial products based on the entomopathogenic fungi listed in Table 11.1 can be found advertised on the Internet. De Faria and Wraight (2007) conducted a survey in 2006 and identified 129 active mycoinsecticide products; while another 42 had been developed since the 1970s but were not commercially available at the time of the survey. Mascarin and Jaronski (2016) subsequently identified over 67 products based on just one species, *Beauveria bassiana*. In addition, many countries, such as Ecuador, have many small enterprises producing mycoinsecticides on an artisanal basis. The status of microbial insecticides, including fungi, in some regions, was covered in a series of articles in the July 2019 issue of the Journal of Invertebrate Pathology (e.g., Arthurs and Dara, 2019; Glare and O'Callaghan, 2019; Hatting et al., 2019; Kumar et al., 2019). Biopesticide marketing surveys indicate a substantial increase in commercial use in the next 5 years, with a compound annual growth rate of 15.8% to reach $4.6 billion in 2025 Markets and Markets (2021). Of course, this prediction includes all insecticidal microbials not just fungi, but fungi are a major component with the same predicted growth rate.

A common characteristic of the entomopathogenic fungi is that, with the exception of the Microsporida, they infect their hosts percutaneously, not perorally. With all but the Lagenidiales and Peronosporomycetes, the infectious stage is passively dispersed; the former two groups have motile zoospores that actively seek out their aquatic hosts. The life cycle begins when the spore contacts the arthropod cuticle, attaching initially by Vander Waals forces, but then adheres more firmly and germinates within a few hours. A penetration hypha is produced and, in some species, an appressorium or anchoring structure. The hypha penetrates the arthropod cuticle by means of several enzymes and mechanical pressure. Once in the hemocoel, the fungus proliferates by means of yeast-like bodies (hyphal bodies in Entomophthorales, blastospores and mycelium in Ascomycetes, mycelium in Oomycetes). As the host dies the fungus rapidly transforms

*Mention of trade names or commercial products in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the author.

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TABLE 11.1 Commercial mycoinsecticide products available in 2007.

Species	Number of products	Percent
Beauveria bassiana s.l.	45	37.2%
B. brongniartii	5	4.1%
Metarhizium anisopliae s.l.	44	36.4%
M. acridum	3	2.5%
Cordyceps fumosorosea	7	5.8%
C. farinosa	1	0.8%
Akanthomyces longisporium	2	1.7%
Akanthomyces muscarium	3	2.5%
Akanthomyces sp.	10	8.3%
Hirsutella thompsonii	1	0.8%
total	121	

Source: Adapted from De Faria, M., Wraight, S., 2007. Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43: 237–256.

into mycelium, and under ideal conditions, particularly an extended period of high humidity emerges to conidiate on the exterior of the insect.

11.2 Production methods for the important insect pathogenic fungi

Mass production methods for the entomopathogenic fungi up to 1988 were reviewed in detail by Bartlett and Jaronski (1988) and the reader is referred to that publication. There have been a few general review publications, namely Feng et al. (1994) and Jenkins and Goettel (1997), as well as many specific studies about some aspects of mycoinsecticide mass production, particularly in the last 10 years. The previous version of this chapter (Jaronski, 2013) detailed the "state of the art" to that time. The present chapter seeks to continue the update of the older literature, delineate important aspects, and identify areas of needed research.

11.2.1 Lagenidium giganteum

Lagenidium giganteum (Schenk) (Oomycota: Lagenidiales) is the principal species studied for development, with larval mosquitoes as its principal target. A key aspect of this fungus is that the infectious stage is a motile zoospore that actively seeks out hosts and is produced by either mycelium or from germinated resting spores. Elution of nutrients from the mycelium and any carrier matrix, for example, agar medium, is required for zoospore production from competent mycelium. Oospores, sexual resting bodies, can also produce zoospores upon rehydration, although the cues for relatively synchronous zoosporogenesis are unclear. Thus, efficient production of shelf-stable formulations is difficult. For a review of *L. giganteum* biology see Kerwin (2007).

Exogenous sterols, specifically cholesterol, ergosterol and campesterol, are essential nutrients for the production of both zoosporogenic mycelium (Domnas et al., 1977) and oospores (Kerwin and Washino, 1983). Attenuation of the zoosporogenic capability follows culture without these sterols (Lord and Roberts, 1986). Initially, the production of zoosporogenic mycelium used agar-based media, primarily for experimental use (Jaronski et al., 1983). Subsequently, May and VanderGheynst (2001) developed a solid substrate medium. This medium consisted of wheat bran supplemented with cholesterol, peptone, autolyzed yeast extract, glucose, corn oil, and lecithin, and was inoculated with a liquid preculture. Simpler media were subsequently developed by Maldonado-Blanco et al. (2011), who identified soybean meal and sunflower meal as suitable sources of the required sterols. Little work has been done since.

On a commercial scale, *L. giganteum* mycelium and oospores were produced in liquid fermentation using media consisting of crude carbon and nitrogen sources, with vegetable or fish oils to provide the required sterols and

unsaturated fatty acids (Kerwin and Washino, 1986); the unsaturated fatty acids, primarily triglycerides, were thought to help solubilize the sterols to optimize uptake and provide a higher percentage of fatty acids increasing zoospore production (Kerwin and Washino, 1986). Another critical component is $CaCl_2{-}2H_2O$. Fermentation output at pilot-scale level was 1−5 L fermenter volume ha^{-1} mosquito habitat, with a production cycle of 3−4 days (Kerwin and Washino, 1987). Commercial production used this method, with harvesting of the fungus from liquid medium and storage in refrigerated containers; effectiveness of such material lasted 1−3 weeks (Vander-Gheynst et al., 2000). Kerwin (2007) still considered economical scale-up of liquid fermentation a serious challenge. Although mycelium and oospore formulations of the fungus were registered and commercialized by Agraquest Inc. in the US, the company abandoned the continued sale of the fungus. No further effort on a commercial scale has occurred. As an alternative, *L. giganteum* could be produced using wheat bran as a solid fermentation substrate (Vander-Gheynst et al., 2000). The fungus retained its efficacy for 4 weeks. Glucose and wheat germ oil could increase the shelf life of the fungus. The whole culture could be efficacious against larval mosquitoes, at least in laboratory assays.

11.2.2 Leptolegnia chapmani

Leptolegnia chapmani Seymour (Straminipila: Peronosporomycetes) has been under study for several years as an alternative mosquito control agent (Zattau and McInnis, 1987). Much less work has been done regarding production of *L. chapmani*, primarily because the fungus remains in an experimental mode. Pelizza et al. (2011) evaluated a series of agar-based media for zoospore production. Although they observed most media supported mycelial growth equally, zoospore production in an agar medium that contained 10% Fortisip, a complex human nutritional supplement, was 10-fold greater than any of the other media. The sterol requirement of *L. giganteum* does not seem to exist for *L. chapmani*.

11.2.3 Coelomomyces spp. Keilin

This group of more than 70 species, within the Phylum Chytridiomycota, Order Blastocladiales, are aquatic, obligatory pathogens of aquatic Diptera, including Culicidae, Psychodidae, Chironomidae, Simuliidae, and Tabanidae (Chapman, 1985; Whisler, 1985). These fungi are also notable for alternating sexual and asexual stages and involving copepods or ostracods as alternate hosts. Even though the *Coelomomyces* species have been observed to be very effective control agents of mosquitoes the dependence on in vivo production has greatly limited their usefulness as inundative biocontrol agents (Scholte et al., 2004).

11.2.4 Entomophthorales

Previously classified within the Zygomycetes, these fungi have been placed in a new subphylum, Entomophthoromycotina, representing a monophyletic lineage distinct from all the other fungi (Humber, 2012). Most species within this group are insect pathogens, with a few species also attacking nematodes and mites. A notable characteristic for most species is a degree of high host specificity. Many species have never been cultured in vitro because of their nutritional fastidiousness.

A major impediment in developing Entomophthorales, even for efficient inoculative biocontrol efforts, much less inundative applications, has been their biology. The conidia are short-lived after in vivo production and extremely difficult to produce efficiently in vitro. In vivo, hyphal bodies, or protoplasts, are the basic vegetative stages of these fungi. Resting spores (azygospores, and zygospores in some species), have, however, been deemed to be the more promising infectious propagule for biocontrol efforts. In *Zoophthora radicans* (Brefeld) Batko and other species the azygospores are formed in vivo by the proliferative hyphal bodies in the insect's hemolymph. These azygospores are generally dormant after production and are typically in the overwintering stage in temperate climates. Under specific environmental conditions, the resting spores germinate to produce primary conidia, which then give rise to infectious conidia, which then continue the fungus life cycle by infecting insect hosts. In limited field introductions, azygospores have been typically produced in vivo, being collected from lab-infected cadavers or from a soil matrix in which the infected insects have died. Neither process is efficient or amenable for large-scale introduction (Hajek, 1997). Laboratory methods for isolating and culturing these fungi are thoroughly discussed by Hajek et al. (2012).

Bartlett and Jaronski (1988) reviewed the status of entomophthoralean production up to that time. Some limited research into mass production of Entomophthorales had been conducted prior to 1984, namely efforts by Latge (1975, 1978), and Latge (1981). Since then, there has been some effort beyond laboratory level media to better study a particular species, but only a few studies attempting to mass-produce an entomophthoralean fungus. A review of the literature

reveals that only the species listed in Table 11.2, have been mass-produced in vitro, all on only an experimental level. There has not been any commercial production of an Entomophthorales to date

Culturing entomophthoralean fungi in vitro varies widely in difficulty and depends on the species and even the isolate. In general, *Neozygites* Witlaczil species are among the more difficult species to grow, and *Conidiobolus* (Costantin) Batko the easiest. The earliest advances in the in vitro production of the Entomophthorales were use of Grace's insect cell culture medium supplemented with fetal bovine serum (5% v/v), which simulated the insect hemolymph (Dunphy et al., 1978). This approach was subsequently used by others, such as Kogan and Hajek (2000), who produced azygospores of *Entomophaga miamaiga* Humber, Shimazu & Soper with this medium. Alternatively, some species could be grown on Sabouraud dextrose agar supplemented with egg yolk and milk, or coagulated egg-yolk-milk medium (Hajek et al., 2012). However, neither approach is practical, much less economical, for any level of mass production. Beauvais and Latge (1988) advanced the art with a medium of glucose, yeast extract, lactalbumin hydrolysate, NaCl, and 10% fetal bovine serum (GLEN medium). GLEN and modified Grace's Medium have also been used to grow *Entomophthora thripidum* Samson, Ramakers & Oswald in the laboratory, but not on a large scale (Freimoser et al., 2003).

Nolan (1993) initially devised a defined medium, which allowed growth in both stationary and shaken cultures in the absence of fetal calf serum and was developed for protoplasts of the fungus *Entomophaga aulicae* (Reichardt) Humber. The protein "requirement" was obviated and growth was enhanced by the addition of hematin (0.5 pg/mL) and oleic acid (1.0 pg ml^{-1}). Nolan (1993) developed a low-cost liquid medium for production of *E. aulicae* hyphal bodies competent to form conidia. The medium consisted of a basal medium of trace salts, amino acids, sucrose, glucose and buffer, plus 0.8% tryptic soy broth and 0.4% calcium caseinate and supported the growth of several isolates. Inoculant protoplasts were grown on "traditional" Grace's tissue culture medium supplemented with 5% fetal bovine serum.

Hyphal bodies of *Neozygites floridana* (Weiser & Muma) Remaudier & Keller were produced in vitro using Grace's cell culture medium plus 0.33% lactalbumen hydrolysate and 0.33% yeastolate (Liete et al., 2000). Yields were over 10^6/mL. These hyphal bodies would produce primary conidia from which the infectious capilloconidia could be generated. Leite et al. (2005) refined the medium for *N. floridana* further, using glucose, skim milk, yeast extract, peptone, and trace salts, but the complexity of preparing this medium may preclude its use in large volume.

In seeking a mass-production medium for *Z. radicans* azygospores, Senthilkumar et al. (2011) tested a series of concentrations and ratios of sunflower oil or dextrose as a carbon source and a yeast extract or peptone as a nitrogen source

TABLE 11.2 Entomophthorales species that have been mass-produced in vitro.

Species	References
Pandora nouryi	Zhou and Feng (2009)
Pandora (Erynia) neoaphidis	Hua and Feng (2003), Uziel and Kenneth (1991), Shah et al. (2005)
Zoophthora radicans	Senthilkumar et al. (2011), Pell and Wilding (1992)
Pandora delphasis	Uziel and Kenneth (1991)
Entomophaga aulicae	Nolan (1993), Nolan (1993)
Entomophthora thripidum	Freimoser et al. (2003)
Entomophthora maimaiga	Hajek and Plymale (2010)
Batkoa sp.	Leite et al. (2005)
Furia sp.	Leite et al. (2005)
Neozygites floridana	Liete et al. (2000), Leite et al. (2005), Delalibera et al. (2003)
Entomophthora (Conidiobolus) coronata	Wolf (1951)
Entomphthora obscura	Latge (1975)
Neozygites tanajoae	Delalibera et al. (2003)
Entomophaga grylli pathotype 1	Sanchez-Pena (2005)
Neozygites parvispora	Grundschober et al. (2001)

in a liquid medium and discovered the optimal was a 4:8 yeast extract: sunflower oil medium. Alternatively, *Z. radicans* could be produced as mycelium in a liquid culture medium of yeast extract, dextrose and sunflower oil, which mycelium was then harvested by filtration, washed and prepared as a thin slurry. The slurry was treated with 10% maltose, matured at 4°C, and then air-dried (McCabe and Soper, 1985; Wraight et al., 2003). This dry, marcescent mycelium was then prepared as a granular formulation, which when rehydrated and exposed to the appropriate environmental conditions would generate large numbers of the infectious conidia. The dry mycelium formulation produced equivalent or greater numbers of conidia than fungus on leafhopper cadavers. However, Li et al. (1993) discovered that dried mycelium preparations of *Z. radicans* and *Erynia (Pandora) neoaphidis* Remaudier & Hennebert did not survive milling or freezing. The production process is complex and imposes serious constraints on mass production especially for inundative releases of this fungus. The process could be feasible for inoculative release in order to establish epizootics, but has still not been capitalized upon. One impediment, at least in the US, Canada and the EU, is that any use of a microbial to control an insect is subject to registration with the respective regulatory authorities, and inoculative release may not be a sufficient commercial incentive. Latge et al. (2004) described the best media for *Entomphthora virulenta* Hall and Dunn zygospores consisted of dextrose and corn syrup as carbon sources, and yeast extract, soybean flour, or cottonseed flour as the best nitrogen sources. The resulting zygospores had a 70% germination rate. A liquid culture medium for *E. neopahidis* was determined by Gray and Markham (1997) to consist of glucose, yeast extract, mycological peptone, KH_2PO_4, Na_2HPO_4 and 0.01% oleic acid in 1.5 L fermentation volumes. They obtained considerable mycelial biomass in batch but not continuous fermentations. A disadvantage was that large inoculum volumes were necessary to detoxify an essential nutrient, oleic acid.

Media for *Batkoa* (Keller) and *Furia* (Humber) species were devised by Leite et al. (2005). For *Furia* the best medium consisted of 0.33% each yeast extract, beef extract, and skim milk in a basal medium of 2.66% glucose and trace salts. Although several other combinations of nitrogen sources gave good yields, skim milk was associated with the best media. For *Batkoa*, in contrast, yeast extract was the best nitrogen source, while skim milk and peptone did not seem to be important nitrogen sources for this fungus. Both fungi produced mycelium in these media, mycelium that presumably could be processed and preserved using some modification of the McCabe and Soper (1985) process. The Leite et al. (2005) study illustrates the differences in optimal media that can exist among species of Entomophthorales, especially differences in response to different concentrations of complex nitrogen sources, as well as the sources themselves. The best, simple, in vitro, mass production of an entomophthoralean fungi using a simpler medium has been with *Pandora neoaphidis* (Remaud & Hennebert) Humber, *P. nouryi* (Remaud & Hennebert) Humber and *Z. radicans* on broom corn millet (Hua and Feng, 2003, 2005; Zhou and Feng, 2009). Autoclaved broomcorn millet (with 36% moisture content) supplemented with Sabouraud dextrose broth and inoculated with the fungus evidently colonized the grains, which then served as surrogate aphid cadavers, allowing subsequent sporulation that was 2−3 times more abundant and for a greater duration than in aphid cadavers.

There have been several efforts of in vivo production. Mullens (1986) devised a method to infect large numbers of houseflies with *Entomophthora muscae* (Cohn) Fresenius. By this method several hundred adult flies are exposed to conidial showers from infected insects in a small container, ensuring good dose transfer. Serial repetitions of these exposures could greatly increase the number of infected insects, which could then be released alive. Carruthers et al. (1997) described using *Entomophaga grylli* (Fresenius) Batko, produced in vivo, in inoculative releases against grasshoppers. The insects were infected by injecting protoplast cultures and were then released live into natural grasshopper populations. The potential for using dried infected hosts (*Plutella xylostella* L. with *Z. radicans* resting spores) was presented by Pell and Wilding (1992). Second instar larvae were exposed to sporulating plate cultures, reared until death but before fungal sporulation occurred and then dried at ambient temperature and 40% relative humidity. Steinkraus and Boys (2005) harvested large numbers of cotton aphid (*Aphis gossypii* Glover) infected with *Neozygites frezenii* (Nowak.) Remaud & Keller from natural epizootics and preserved them by drying the aphid-infested leaves over silica gel. The dried cadavers had a very high level of sporulation when rehydrated, even 6 months later. In this manner, they were able to collect 25,572 infected aphids from 189 leaves. The key to success with such a method, however, is to find an appropriate epizootic, which may not be always possible.

11.2.5 Microsporidia

These organisms have been historically associated with the Protozoa, but have been reclassified within the kingdom Fungi, as the Phylum Microsporidia (Keeling 2009). Nevertheless, their biology and morphology are unique and distinctly different from the other fungi. A spore is an infectious agent, and the infection process most commonly involves the host ingesting the spores, explosive spore germination in the host gut, with insertion of the nucleus and associated

cytoplasm into a gut epithelial cell through a thin, hollow, polar filament rapidly everted from the spore. The microsporidian then develops within the host cell. Vertical transmission via transovarial and transovum routes are also common among the microsporidia, as are intermediate hosts. Hosts of microsporidia span a wide range, from Protista to warm-blooded vertebrates, but in general, each species is somewhat host-specific. See Solter et al. (2012a) for a review of microsporidian biology. One species, *Paranosema* (*Nosema*) *locustae* (Canning), has been registered as a microbial pesticide and commercialized in the US for the control of grasshoppers (US Environmental Protection Administration, 2000). In general, however, biocontrol efforts with microsporidians have focused more on inoculative or augmentative approaches where smaller amounts of infective spores are needed than in inundative use.

A salient feature of microsporidia is their obligate intracellular development. Thus, these organisms can be mass-produced only in vivo, in their hosts (Henry, 1985) or in invertebrate cell culture (Visvesvara et al., 1999). For example, *P. locustae* is produced by perorally infecting grasshoppers with a dose that is optimal for spore production, allowing the pathogens to multiply within the host, fragmenting the cadavers in a mill and suspending the macerate in water. After the insect parts are removed by filtration, the resulting spore suspension is further purified and formulated with a wheat bran carrier (Henry, 1985). Spores of *P. locustae* can be stored in refrigerated sterile water, lyophilized, or vacuum-dried before formulation. Infected cadavers can also be dried and stored until further processing. The reader is referred to Solter et al. (2012a, 2012b) for further information about mass-production methods for microsporidia.

11.2.6 Ascomycetes Hypocreales

Most of the commercial development of the entomopathogenic fungi has been directed toward this group of fungi. These particular species, once placed in the Deuteromycete (Imperfect) fungi, have now been assigned to the family Clavicipitaceae within the Ascomycete order of Hypocreales, based on their molecular association to teleomorph stages. These fungi include the genera *Beauveria, Metarhizium, Cordyceps* Fries *(*formerly *Isaria and before that Paecilomyces)*, *Akanthomyces* Liebert *(*formerly *Lecanicillium and before that Verticillium)*, and the species *Hirsutella thompsonii* Fisher, *Nomuraea rileyi* (Farlow) Samson (now *Metarhizium rileyi* (Farl.) Kepler, Rehner & Humber), *Aschersonia aleyrodis* Webber, *Culicinomyces clavosporus* Romney and Rao, *and Tolypocladium cylindrosporum* (Gams.) A word needs to be made about recent nomenclatural changes of several prominent entomopathogenic fungi. The species are formerly known as *Isaria fumosorosea* (Wize) and *I. farinosa* ((*Holmsk.*) *Fr.*), are now in the genus *Cordyceps* (Kepler et al., 2017), and at least some strains previously considered *C. fumosorea*, such as the commercial Apopka97, have been shown to actually be *C. javanica* (Frieder & Bally) Kepler, B. Shrestha & Spatafora (Ayala-Zermeño et al., 2015; Gallou et al., 2016). The genus *Lecanicillium* has been renamed *Akanthomyces* (Kepler et al., 2017). What had been considered *B. bassiana* for decades has been revealed to be a complex of species, some of which have not yet been erected as formal species (Rehner et al., 2011; Bustamante et al., 2019). There seem to now be 31 species of *Beauveria* (National Center for Biotechnology Information NCBI, 2021a). Likewise, *M. anisopliae* sensu lato has been split into 52 species (Mongkolsamrit et al., 2020; National Center for Biotechnology Information NCBI, 2021b) and additional species are expected with further molecular analyses. The fungus formerly known as *Nomuraea rileyi* is now *Metarhizium rileyi* Kepler, S.A. Rehner & Humber (Kepler et al., 2014). These changes add a layer of complexity, possibly accompanied by species differences in important phenotypic characteristics, for example, tolerance to UV and inherent shelf life of propagules. These nomenclatural changes make generalization difficult. Also, the inherent quantitative ability to produce propagules (aerial conidia, blastospores, microsclerotia) is strain-specific, so published observations about propagule yields for one strain may not be reflected in other strains of the same species. This disparity among strains of a species has been demonstrated several times, for example, Mascarin et al. (2015b).

For reviews about the biology of the three most important genera, see Zimmermann (2007a) for *B. bassiana s.l.*, Zimmermann (2007b) for *Metarhizium*, and Zimmermann (2008) for *Isaria* (*Cordyceps*). Each of these references contains considerable information about the biology of the respective genera.

In general, these fungi have four major propagule types that can be used. In nature, the aerial conidium is the primary infectious propagule. These are the spores that are produced on the exterior of fungus-killed insects. Blastospores are the proliferative stage within the insect for many of these fungi and can also be produced in liquid fermentation., This ability is not surprising if one considers an insect as merely a six-legged flask of culture medium. Under certain liquid fermentation conditions, mainly substitution of inorganic for organic nitrogen, *Beauveria* spp. and *Metarhizium* spp. can produce "microcycle" conidia (Thomas et al., 1987; Bosch and Yantorno, 1999; Zhang et al., 2009). These conidia are not true conidia and are produced on the ends of hyphal strands. Lastly, mycelium, the major form of fungal vegetative proliferation, or mycelial derivatives such as the microsclerotia, can be used. But in this last case, the mycelium or its derivatives are really producers of conidia—a way to deliver conidia to the insect.

Because of the significance of species within the genera *Beauveria* and *Metarhizium* among the entomopathogenic Ascomycetes, emphasis on developments for their mass production follows. Specific production methods developed for *Cordyceps*, *Hirsutella*, *Aschersonia*, *M. rileyi*, *Akanthomyces*, and *Culicinomyces* are subsequently reviewed.

11.2.6.1 Solid substrate fermentation

The fundamentals of solid substrate fermentation to produce entomopathogenic fungi were discussed by Jaronski and Jackson (2012) and the reader is referred to that publication. A relatively recent overview of solid substrate fermentation systems is presented by Costa et al. (2018), and Krishania et al. (2018). Descriptions of practical small-scale methods, yielding decagrams, even hectograms, of conidia per kg of the substrate may be found in Jaronski and Jackson (2012). Older descriptions of solid substrate production systems are presented by de Aquino et al. (1977), Cherry et al. (1999), Pérez-Guerra et al. (2003), Bateman (2007). More recently Méndez-González et al. (2018b), and da Cunha et al. (2019) describe relevant systems. Physiological aspects are discussed by Loera-Corral et al. (2016).

11.2.6.1.1 The end products of solid substrate fermentation

Solid substrate fermentation, mimicking the natural conidiation processes, yields aerial conidia as the final product (Fig. 11.1). Aerial conidia are the natural infectious bodies for these Ascomycetes. In a few isolated cases, the fermentation is terminated after the fungus has penetrated the nutritive substrate but before conidiation has begun (Kirchmair et al., 2007; Skinner et al., 2012). This process yields dried grain kernels colonized by *B. bassiana* or, in particular, *M. anisopliae*, which remain competent for regrowth and sporulation upon rehydration. This rehydration occurs, for example, after such granules are applied into the soil by a sowing machine in combination with a rotary harrow (Kirchmair et al., 2007) or mixed into horticultural soil (Skinner et al., 2012), creating focii of conidia within the habitat of the target insect.

11.2.6.1.2 Substrates and media

A wide variety of organic materials have been evaluated as substrates for the Ascomycetes. While rice and barley seem to be the major substrates used in the Tropics and the Northern Hemisphere, respectively, there has been considerable effort to identify low-cost agricultural materials especially byproducts and waste products as suitable substrates. Such efforts have been particularly frequent in India, Pakistan, and China. Table 11.3 lists some of these alternative substrates. There have been no substantive updates since the previous version of this chapter (Jaronski and Jackson, 2012). Comparisons, especially among different reports, are very difficult because of different fungus species and strains, different fermentation conditions, and different supplementations. A few studies have quantitatively examined certain, basic nutritional aspects, such as C:N ratio (Ortiz-Urquiza et al., 2010) or controlled levels of carbohydrates (Domenech et al., 1998). Nevertheless, rice remains the substrate of choice where it is readily and cheaply available. In North America and Europe, barley, particularly dry, flaked barley, seems to be preferred because of its superior handling characteristics and cost relative to rice (Jaronski and Jackson, 2012).

Several inorganic substrates have been identified in recent years. These include granules of calcined diatomaceous earth (diatomite), for example, Celetom MP, Solid-a-Sorb or Floor-Dry in the US, (Jaronski and Jackson, 2012; Crangle

FIGURE 11.1 (Left) Plastic, vented spawn bag with 200 g of well-sporulated *Metarhizium anisopliae* culture, from the author's laboratory. This bag produced 31 g of harvested dry conidial powder with a titer of 5.2×10^{10} viable conidia/g. (Right) Solid substrate (flaked barley) with well sporulated *Beauveria bassiana*. Note the abundant conidia in the form of powder on the substrate. This 1 kg (dry weight) culture of *B. bassiana* yielded 105 g dry conidial powder with a titer of 1.4×10^{11} viable conidia/g.

TABLE 11.3 Some solid substrates evaluated for production of the principal entomopathogenic ascomycetes.

Substrate	Fungus
Ag byproducts	*Metarhizium*
Bagasse ± 2% dextrose	*Beauveria*
Barley	*Beauveria*
Beetroot	*Beauveria*
Broken rice	*Metarhizium*
Broken rice + CaCl2	*Beauveria, Metarhizium*
Carrot tubers	*Beauveria*
Cassava chips	*Metarhizium*
Chickpea	*Metarhizium*
Coconut cake	*Beauveria*
Cottonseed cake	*Beauveria*
Finger millet	*Beauveria*
Grade 3 unpolished rice	*Metarhizium*
Groundnut cake	*Beauveria*
Kodo millet	*Metarhizium*
Maize	*Beauveria, Metarhizium*
Maize bran ± 2% dextrose	*Beauveria*
Mijo grains + organic N	*Nomuraea*
Millet	*Beauveria, Metarhizium*
Neem cake	*Beauveria*
pearl millet	*Beauveria*
Potato tubers	*Beauveria*
Prawn waste	*Beauveria*
Press mud ± 2% dextrose	*Beauveria*
Rice	*Metarhizium*
Rice + saccharomyces	*Beauveria, Metarhizium*
Rice bran ± 2% dextrose	*Beauveria, Metarhizium*
Rice Flour	*Metarhizium*
Rice hulls, sawdust:rice bran ± 2% dextrose	*Beauveria*
Rice husk ± 2% dextrose	*Beauveria*
Sesame cake	*Beauveria*
Sorghum	*Beauveria, Metarhizium*
Soyabean	*Beauveria, Metarhizium*
Sugarcane bagasse ± yeast, molasses	*Beauveria, Metarhizium*
Sugarcane press mud	*Beauveria*
Tapioca rind	*Beauveria*
Tapioca tubers	*Beauveria*

(*Continued*)

TABLE 11.3 (Continued)

Substrate	Fungus
Wheat	*Beauveria, Metarhizium*
Wheat bran + $Al(SO_4)_3$	*Beauveria*
Wheat bran + organic N	*Nomuraea* (now *Metarhizium*)
Wheat bran ± 2% dextrose	*Beauveria*

2011; Wikipedia contributors, 2021), and open-pored clay granules, for example, Seramis (Seramis GmbH). The use of clay granules to produce *B. bassiana* is described in detail by Desgranges et al. (1993) and Guillon (1997). Diatomite can be obtained in a range of sizes from several mm in diameter to a coarse powder, has a high surface to volume ratio and can absorb aqueous liquids up to 110%−140% of its weight. Seramis is a processed, particulate clay composed of kaolinite, illite and quartz, produced in the Westerwald region of Germany, and used primarily in hydroponics, plant bedding and interior landscaping industries in Europe. It, too, is open-pored and highly absorbent (Ecoflora, 2021). Unlike cereal grains or other organic materials, mineral carriers have the advantages of allowing flexible control of nutrients tailored for each fungus species and strain, and the ability to be recycled after washing and sterilization. This ability for recycling avoids disposal issues, which can be considerable, as is faced by one North American company having 10,000 kg production runs. In the 1980s this author developed a pilot-scale (100 kg batch size) process using Celetom granules with a liquid medium to produce $5-7 \times 10^{12}$ *B. bassiana* ARSEF252 conidia/kg substrate (Jaronski, unpublished data). If harvesting was performed aseptically, it was possible to obtain a second round of conidiation after the first was removed and the substrate reincubated. A third flush became contaminated, however, with *Aspergillus*, and in general, such practice is not recommended for a quality product. Used Celetom substrate could be reused after resterilization, on a large-scale using counter-current heating, and reinoculation with liquid preculture. Niedermayr et al. (2012) noted that an unidentified, open-pored clay granule produced only 10% of the spore yield of *M. brunneum* BIPESCO5 (also known as Met52) than on several grains. Their comparison was flawed, however, because they used a packed bed column fermenter for the clay granules and plastic bags for the grains. Further, they did not optimize the liquid medium for the clay granules. The grain in bags yielded more typical yields for this fungus. Hemp-based animal litter (Hemparade, HempFlax bv., The Netherlands) has also been used as an inert carrier, impregnated with a nutrient medium (Breukelen et al., 2011). The hemp evidently afforded more than adequate porosity for good gas and heat transfer in a packed-bed, column fermenter. Conidia could be removed from crushed, dried whole culture by simple mechanical classification. While yields were excellent, comparable to those on barley in vented mushroom spawn bags (Jaronski, unpublished data), the cost of the substrate and nutrients was not revealed. In addition, hemp does not seem to be recyclable. Another novel substrate is Amberlite IRA-900 ion exchange resin impregnated with nutrient medium, which has been used for *Aspergillus niger* van Tieghem, but has not yet been assessed for any of the entomopathogenic Ascomycetes (Auria et al., 1990).

11.2.6.1.3 Equipment for fermentations

By and large, most academic and industrial systems have used "traditional" solid substrate fermentation, either manually intensive or highly mechanized, for producing these Ascomycetes, with a few systems employing submerged liquid fermentation for specific species. In situations where labor costs are low, allowing labor intensive approaches, polypropylene bags are used. These bags include specialized, vented mushroom spawn bags, such as Unicorn spawn bags (Unicorn Industries, Plano TX) and SacO2® microsacs (Combiness, Belgium), zipper-lock bags, and simple shopping bags (Jaronski and Jackson, 2012). Examples are shown in Fig. 11.2. A unifying characteristic is that the different high-density polypropylene plastic bags are autoclavable; unused, unopened, zipper-lock bags are often sterile inside so do not be autoclaved. Certain production operations in Africa utilized plastic bags for the initial mycelial colonization and growth, then transferred the cultures to open, nonsterile, plastic laundry hampers or tubs for the sporulation phase within a controlled environment (Cherry et al., 1999; Bateman, 2007). *Metarhizium* production in Brazil since the 1970s has involved solid substrate in plastic bags (Aquino et al., 1975; Mendonca, 1991) as has *Metarhizium* production in Nicaragua (Grimm, 2001). Fig. 11.3 illustrates *B. bassiana* mass production using plastic bags in Latin America, while Fig. 11.4 shows the highly mechanized production system developed by Mycotech Corp. in the 1990s.

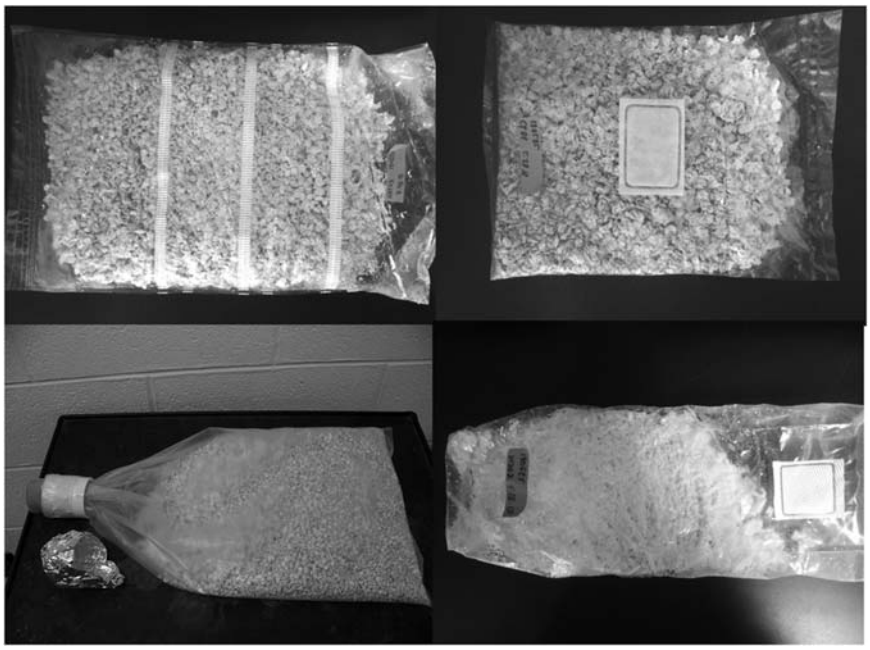

FIGURE 11.2 Different types of plastic bags used for solid substrate production of Ascomycete conidia as used in the author's laboratory. (A) A European mushroom spawn bag containing 2 kg substrate (flaked barley). The white ribbons are microscopic pore vents for gas exchange. (B) An American-made spawn bag with a single 0.5-micron pore vent for gas exchange, containing 500 g substrate. (C) Another style of American vented spawn bag, also with 500 g substrate. (D) Plastic autoclave bag fitted with a PVC pipe joint and foam plug to allow gas exchange and filled with 1 kg substrate.

FIGURE 11.3 Typical plastic bag-based mass production of *Beauveria bassiana* as is practiced in many countries. *Photograph courtesy Miguel Rincon Najera.*

FIGURE 11.4 The 1990s Mycotech Corp. solid substrate mass production system for its *Beauveria bassiana*. (Left) Fermentation tanks for the production of inoculum for subsequent solid substrate phase; (Center) Two fermentation chambers (note large shovel for scale); (Right) Harvested *B. bassiana* conidia from one fermenter run, totaling 232 kg conidia powder with a titer of 1.2×10^{11} conidia/g.

Open trays of the inoculated substrate have also been employed (Alves and Pereira, 1989; Claro, 2006). The technology underlying such systems is the Koji tray fermentation whereby the inoculated substrate is a shallow bed in a tray with perforated bottom and open or mesh-covered cover. Ye et al. (2006) described an upright incubation chamber containing 25 mesh-bottom, open trays, each with the capacity of 2 kg rice. The chamber had a substrate volume of

0.72 m^3 and occupied a surface area of 0.36 m^2, and, while temperature could not be regulated, humidity could be controlled during the fermentation. With such a chamber, fully loaded, they obtained 2.4×10^{12} *B. bassiana* conidia/kg substrate. A similar tray system within an incubator was described by Mendez-Gonzalez et al. (2018a). Here, 25 porous-bottomed trays, each with 2−3 kg rice substrate arranged in a 4 cm deep bed, were fitted in parallel into a $60 \times 60 \times 200$ cm chamber. A key to good aeration was the porous, as opposed to the solid, bottom of each tray, and steady aeration of the chamber with filtered, humidified air. Substrate dehydration during the fermentation is a concern, however, as well as the generation of metabolic heat during the vegetative growth of a fungus. da Cunha et al. (2019) also described a tray system suitable for evaluating preferred temperature and aeration regimes. Their findings point out the exothermal nature of fungal solid substrate fermentation, and deficiencies of plastic bags in this regard. With trays, the maximum bed depth for good heat transfer and gas exchange was 6−8 cm, even in a forced-air system. Because of the exothermic nature of the fermentation, core temperature of a 6 cm deep bed was 4°C., above ambient, but the surface was at ambient. With vegetative growth and sporulation impeded above 32°C. for most strains in the genera *Beauveria, Metarhizium, Cordyceps*, the ambient temperature must be controlled for optimal spore production. A thinner bed, 2−4 cm, presents much less of a problem, but a thinner bed means more trays for a given amount of substrate.

Several groups have further explored packed bed fermentation technology based on early research. Since the review of Bartlett and Jaronski (1988), there has been considerable development in such systems. Early, comprehensive reviews of these systems are presented by Durand (1998, 2003), Durand et al. (1996), Raimbault (1998), and Krishna (2005), and will not be discussed further here.

Significant development of commercial equipment is the Prophyta packed bed fermenter, patented by Luthe and Eiben (2003) and described in detail by Eiben and Lüth (2006). This fermenter is illustrated in a company brochure as shown in Fig. 11.5 (Prophyta, 2012), problems with packed, aerated beds include uneven air flow through the substrate creating pockets of overheated, anaerobic conditions and pockets of the substrate with moisture that are suboptimal for fungal growth, even with water-saturated air supply. The packed bed fermentation research cited here was conducted with columns only 2−5 cm in diameter. For significant-scale, practical production a large number of such columns would be needed, presenting a commensurate workload, esp., in filling columns with the inoculated substrate, as well as emptying them to harvest the conidia. Alternatively, larger packed-bed systems can be employed but these masses are subject to serious uneven air flow as encountered by Underkofler et al. (1947). Use of packed bed fermentation for entomopathogenic fungi has been discussed most recently by Mendez-Gonzalez et al. (2018a), Méndez-González et al. (2018b), Méndez-González et al. (2020), Méndez-González et al. (2021), and da Cunha et al. (2019).

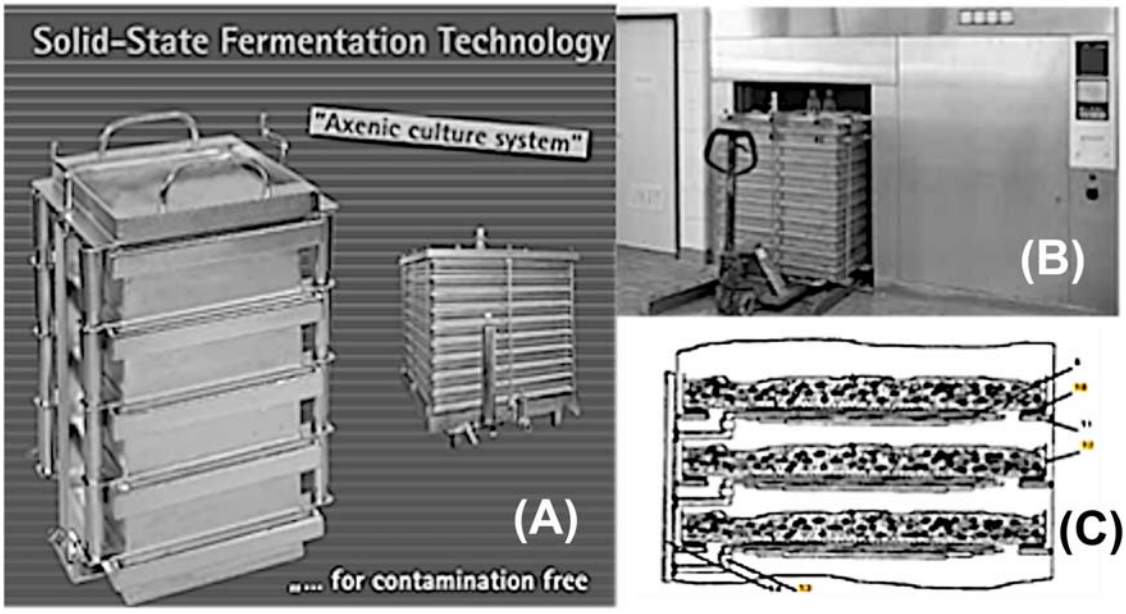

FIGURE 11.5 Stacked packed bed fermenter as developed by Peter Lŭth and his company, Prophyta Gmbh., in Germany in the early 2000s. (A) Images of the laboratory/pilot and production scale fermenters from a company brochure; (B) the production scale Prophyta fermenter unit ready for autoclaving; (C) a diagram of the aerated beds within the fermenter. *(A and B) From Prophyta (2012) and (C) from Luthe and Eiben (2003).*

Sterilization of a large mass of substrate is a challenge. With plastic bag systems, the substrate is easily sterilized by autoclaving each 100−500 g amount in its plastic bag. A major advance in large-scale solid substrate fermentation of edible fungi that is relevant here has been the development of a steam sterilized, double cone blender, holding several hundred kilograms of substrate (Maul et al., 1980). The device allows in situ steam sterilization of substrate, controlled cooling, and subsequent thorough mixing of added liquid inoculum with the substrate. A chute at the pointed end of the V allows for aseptic filling of spawn bags of other containers in an efficient, rapid manner. This system is in use not only by a large North American mushroom spawn producer, but also by several other entities to produce atoxigenic *Aspergillus flavus* Heinrich and Link, and mycoinsecticides. It overcomes a major impediment to very large-scale production—efficient preparation and sterilization of a large amount of substrate. One model is available from Chinese sources (Lianyungang Guoxin Mushroom Complete Equipment Co., Ltd., 2021). An alternative is a continuous, segmented-flow, aseptic processing system designed for food products (Stephens and Walker, 2003; Anderson and Walker, 2005).

11.2.6.1.4 Fermentation parameters: (inoculum, moisture, temperature, aeration, pH)

Strictly speaking, the most efficient solid substrate fermentation is really biphasic, with an initial step being a liquid fermentation to produce inoculum, the most common practice, although some researchers and enterprises use an aqueous suspension of conidia to initiate the solid substrate phase. There are obvious advantages to liquid fermentation in producing inoculum for the solid phase. Conidia require 24 hours to fully germinate and begin colonization of the substrate, while a liquid fermentation (blastospores and mycelia) begins immediately. Further, a liquid fermentation phase greatly multiplies the inoculum potential compared to conidia, because an inoculum should contain 10^7-10^8 propagules/mL with 60−100 mL inoculum/kg of grain substrate. Inoculum concentration affects the duration of the solid substrate phase to reach maximum conidial yield. Nuñez-Gaona et al. (2010) developed a model for *B. bassiana* grown on wheat bran substrate amended with sugarcane bagasse to predict the effect of inoculum concentration. Time to produce 1×10^{10} conidia/g substrate could be halved to 148 hours by increasing the conidial concentration from 1×10^6 to 5×10^7 conidia/g dry substrate. In the author's laboratory, vented mushroom spawn bags and flaked barley or oats, can typically yield 100 g harvested dry conidia/kg with *B. bassiana* titers of $1-1.7 \times 10^{11}$ viable conidia/g, while *M. anisopliae, M. robertsii* strains can produce 80−120 g dry conidia/kg with titers of $4-6 \times 10^{10}$ conidia/g (Jaronski unpublished data). Of course, such prolific production is a phenotypic characteristic of the strains. Other strains can be very poor conidial producers, regardless of substrate, and fermentation conditions.

The ideal liquid fermentation should produce mostly, or entirely, blastospores and short hyphae for optimal dispersion of inoculum through the substrate. Abundant mycelial production, especially in balls or clusters, prevents good dispersion and even colonization of the solid substrate. A wide range of media has been used for the liquid fermentation phase. The simplest recipe is dextrose/sucrose as the carbon source and yeast extract as a source of nitrogenous compounds and vitamins; trace salts are not necessary (Cherry et al., 1999; Bateman, 2007; Jaronski and Jackson, 2012). A medium that works very well with a wide range of *Beauveria* and *Metarhizium* isolates consists of 20−30 g/L glucose or sucrose and 15 g/L yeast extract, supplemented with 17 g/L liquid corn steep or 8 g/L corn steep liquor powder, for example, SOLULYS (Roquette Chemical and Bioindustries) (Jaronski and Jackson, 2012). Corn steep evidently stimulates vigorous blastospore production of both fungi, with minimal mycelial formation. The liquid fermentation requires vigorous aeration, which can be achieved using 200−250 rpm for flask production or an air-lift bubbler fermentation vessel for larger quantities. A typical liquid fermentation cycle takes 72 hours with conidia as inoculant, or 48 hours if a blastospore preculture is used. The media specially used for blastospore production are further discussed in Section 11.2.6.2.2.

In all cases, the solid substrate, whether organic or inert, must be hydrated and sterilized. The role of water in the solid substrate fermentation of fungi is discussed in detail by Gervais and Molin (2003). In situations of potential contamination, the substrate can be hydrated with 0.04% H_2SO_4 and 0.097% KH_2PO_4, to inhibit bacterial growth (Jaronski, unpublished data). In some cases, published protocols using rice substrate have used nutritional additives in hydrating the substrate to increase conidial production: 2% dextrose, cane molasses and torula yeast (Calderón et al., 1995), urea, $(NH_4)_2SO_4$ and yeast extract (Domenech et al., 1998), yeast and molasses (Calderón et al., 1990), and sugar molasses (Sene et al., 2010). Nutritional supplements do not increase the yield of barley, oats, or wheat, presumably because these have more nitrogen and micronutrients than polished rice (Jaronski, unpublished data).

The typical duration of the solid substrate phase is 7−14 days. Depending on the nature of the inoculum (conidia or blastospores/mycelia) substrate is colonized by fungus within the first 24−48 hours, after which there is active mycelial proliferation through the substrate. Then, the culture needs to be dried in most cases. During the initial drying process, as a_w begins to decrease through 0.99, there can be a burst of additional conidiation.

Fermentation parameters, particularly in packed bed approaches, have been dealt with in detail elsewhere (Raimbault, 1998; Krishna, 2005; Mendez-Gonzalez et al., 2018a, 2018b) and the reader is referred to these works. An extremely good discussion of the role of moisture in solid substrate fermentation may be found in Gervais and Molin (2003). A critical moisture level is needed for optimal fungal growth and sporulation. In terms of water activity (a_w), the critical level for most *Beauveria* spp. and *Metarhizium* spp. Seems to be 0.97−0.98 (Humphreys et al., 1989, Hallsworth and Magan, 1999; Nuñez-Gaona et al., 2010). Significant interspecific differences in tolerances to a_w exist (Hallsworth and Magan, 1999). They reported optima ranging between 0.99 and 0.97 for *M. anisopliae* and *C. farinosa* (Holmskiold) (*P. farinosus*), while optimal a_w for *B. bassiana* was 0.998. Tarocco et al. (2005) reported that the greatest conidia yield of a *B. bassiana* on rice was obtained at an initial a_w of 0.99.

The amount of water to achieve this level will vary based on the nature of the substrate. For flaked barley substrate optimal moisture is 52%−56% (w/w) before autoclaving (Jaronski and Jackson, 2012). For rice and sorghum, 22%−30% and approximately 75%−76% moisture, respectively, seem to be optimum (Prakash et al., 2008). A researcher investigating the production of a new isolate of either fungus should experiment to determine optimum moisture levels because there are differences among isolates of any species (Jaronski, unpublished data).

Because the solid substrate phase is an active fermentation, not only does oxygen need to be readily available to all parts of the substrate, but carbon dioxide and heat must be drawn off. Even in vented mushroom spawn bags, O_2 falls to approximately 10% and CO_2 rises to 11% in the head space of the bag within 24 hours of inoculation (Jaronski, unpublished data). In bag fermentation, the substrate mass must be less than 6−8 cm thick to allow proper heat dissipation and gas exchange for the greatest conidial production. Additives to create more headspace within the grain substrate have included aluminum silicate (Saroha and Mohan, 2006), sugarcane bagasse (Nuñez-Gaona et al., 2010); and rice bran/husk (Dorta et al., 1996), wheat bran husk (Arcas et al., 1999). Forced-air, packed-bed fermentation, while potentially overcoming heat dissipation and gas exchange problems of still fermentations, often results in zones of poor growth and sporulation, especially if larger masses are employed (see Section 11.2.6.1.3). This aspect was investigated thoroughly by Underkofler et al. (1947). However, as Arzumanov et al. (2005) observed, forced aeration of packed beds may not be absolutely necessary, at least with certain of the Ascomycetes, as long as the bed geometry allows sufficient gas exchange and heat dissipation.

An advance in the empirical identification of environmental variables optimal for spore production with an economy of effort has been the use of the Response Surface Methodology (Prakash et al., 2008; Dhar and Kaur, 2011; Deng et al., 2011; Qiu et al., 2013). With this approach, used in industry, but rarely in academic studies, Response Surface Methodology combines mathematical and statistical techniques to design experiments and identify optimal conditions using a reduced number of experiments; this approach eliminates the limitations and avoids the laboriousness of single-factor optimization in fermentation.

Conidial yields can vary among strains of each fungus species. For example, Arcas et al. (1999) determined that one strain of *B. bassiana* produced three times as many spores as a second strain under identical fermentation conditions. Conidial production of 15 *B. bassiana* isolates ranged from 1.11×10^{11} to 2.25×10^{13} conidia/g initial dry substrate when grown under identical solid substrate fermentation conditions (Jaronski, unpublished data). It has been my observation that, for the most part, conidial yield is genetically determined in both *M. anisopliae* and *B. bassiana* and can be inversely proportional to virulence for insects.

The observations of Kuźniar (2011) and Zhang et al. (2009) that exposure to light enhanced *B. bassiana* growth and conidiation is contradicted by the very high conidial yields in complete darkness obtained by Bradley et al. (1992), and subsequent commercial production of several *B. bassiana* strains in a completely dark fermentation environment. Similarly, while Onofre et al. (2001) stated that continuous illumination gave a 2.5−5 times increase in spore production of *M. flavoviride*, this has not been my experience. Thus, a light requirement may be strain-specific rather than a general phenomenon. On a practical level, a light requirement may be a considerable challenge in even shallow packed bed fermentations. In bag fermentation, where transparent plastic is used to enclose the fermentation, light is more easily supplied but the substrate mass must be thin enough to allow light penetration through the entire mass.

11.2.6.1.5 Downstream processing

For all purposes except immediate use, the conidia produced on solid substrate must be dried down to a moisture content <5% w/w or water activity (a_w) ≤ 0.3 (Bateman, 2007; Jaronski and Jackson, 2012) for the best shelf life and to be easily formulated. This low moisture is necessary for optimal shelf life regardless of whether conidia are formulated or not. Moore et al. (1996) observed that even trace amounts of moisture in the conidia result in foreshortened shelf life. A relatively short shelf life, of a few weeks to a few months depending on the fungus can be obtained by refrigerating whole, sporulated solid substrate within their original fermentation bags.

There are a number of published drying methods: simple opening of plastic fermentation bags, transfer of sporulated substrate to open trays (Bateman, 2007; Claro, 2006) or tabletops (D.W. Roberts, personal communication), transfer to Kraft paper sacks (Jaronski and Jackson, 2012), or use of air-lift devices. In general, *Beauveria* spp. conidia can be dried relatively quickly—within 2−3 days—without loss in viability, while *Metarhizium* conidia require slower drying, 5−9 days (Hong et al., 2000; Jaronski and Jackson, 2012). There is only one study carefully examining the effect of drying temperature and duration of an entomopathogenic Ascomycete (*B. bassiana*) (Li et al., 2008). In that study, the viability of conidia was affected by different drying temperatures and speeds of drying; 5 hours at 35°C had no effect on conidial viability. Effects of drying temperature and speed on the shelf life of formulated as well as unformulated conidia, an important commercial aspect, are not known, however.

Moisture endpoint is best measured using a water activity meter (see Jaronski and Jackson, 2012) but gravimetric moisture analysis is satisfactory. Water activity (a_w) is a measure of the biologically relevant moisture content of an object. The relationship between water activity and moisture content is graphically and algebraically presented for both absorption and desorption isotherms by Faria et al. (2009); the interested reader should refer to this report for more details.

Extreme desiccation of conidia ($a_w < 0.1$), however, can lead to two problems, one affecting proper quality assurance testing and the second a significant problem in the operational use of such dry conidia. This situation stems from imbibitional damage of the plasma membranes within the conidia during improper dehydration. Problems with conidial rehydration were first observed by Moore et al. (1996) and subsequently studied in more detail by Faria et al. (2010). In a series of experiments, Faria et al. (2009) discovered that extreme desiccation of *Metarhizium* (below a_w of 0.3) conidia requires careful rehydration before suspension in an aqueous medium. The use of cold water (≤15°C) with dry conidia resulted in very low germination. Conidia of *B. bassiana* were more resistant to imbibitional damage, with conidia as dry as aw of 0.02 having germination rates at normal levels except when water was 0°C. Proper rehydration of dry conidia can be by exposure of the conidia to a moisture-saturated atmosphere for a minimum of 30 minutes, as described by Moore et al. (1996), or by use of warm (33°C−34°C) water as described by Faria et al. (2009). The use of warm water must be carefully controlled, because, as Xavier-Santos et al. (2011) observed, different isolates of *Metarhizium* responded differently to immersion at 31°C. and all isolates tested were adversely affected by either 45°C. for 60 minutes or prolonged exposure. Under operational conditions, involving more than a few decagrams of conidia, the need for careful rehydration of very dry conidia can pose logistical challenges, especially with *Metarhizium*. Of course, one can carefully observe the drying process and terminate it when the conidia have reached a water activity of 0.3. Emulsifiable oil formulations seem to confer protection from imbibitional damage (Xavier-Santos et al., 2011).

Mechanical separation of conidia from the dried substrate is the predominant method in commercial use. Harvest of dry conidia can be mechanically simple with spore collection using an appropriate vacuum cleaner system, illustrated in Jaronski and Jackson (2012), or a sophisticated cyclone dust collector connected to some sort of agitation system. There are two ways to do this: vibratory sieving (Fig. 11.6) through graded sieves, 10−20 mesh then 80−100 mesh sieves, and mechanical agitation of the substrate to mechanically release conidia and collect them from the air stream using a cyclone dust collector (Fig. 11.7) (Bateman, 2007; Jaronski and Jackson, 2012). Graded vibratory sieves, ideally on a vibratory rather than rotary shaker, can separate out the conidia from a substrate with 60%−90% efficiency depending on the equipment (Jaronski, unpublished data). The food processing industry has a wide range of separators from small, tabletop models, ideal for laboratory settings and available on several Internet sales sites (Fig. 11.6), to industrial-scale

FIGURE 11.6 (Left) Batch harvesting conidia of *Metarhizium anisopliae* from substrate by mechanical classification using 20- and 100-mesh sieves on a vibratory shaker. (Right) Electric, vibratory, powder separator, made for domestic kitchen use but ideal for the small-scale harvest of conidia from the solid substrate, can process 1 kg of dried substrate per 15-minute cycle in the author's laboratory. A 33-cm diameter, 10- or 20-mesh sieve is fitted above an 80- or 100-mesh sieve mounted above a collecting pan. Conidia are collected through the spout at the lower left into a sealed container (plastic bag shown).

FIGURE 11.7 A cyclone dust collector, adapted from the carpentry industry, used to concentrate conidia mechanically dislodged from the solid substrate. The conidia are collected in the barrel beneath the conical collector. There is a HEPA filter on the exhaust to prevent the escape of conidia into the environment.

equipment. One device, using the fluid-bed agitation and cyclone dust collection technology, has been specifically developed for harvesting fungal conidia on both laboratory and production scales (Dropdata Network, 2012, 2017). In one case the conidia are physically dislodged from the substrate in a fluid bed system and in the other, commercial-scale system, in a rotating drum agitator.

Washing the conidia off-dry or semidry substrate has not been pursued to any great extent at least in the published literature. The *B. basiana* production system that I developed in the 1980s employed washing conidia from the moist substrate using cold 0.1 M NaCl, removing nonconidial material with self-cleaning filters, adding a diatomaceous earth filter aid, and then concentrating the conidia with a continuous flow centrifuge or alternatively a ribbon filter. The resulting semidry paste was then rapidly air-dried as a thin layer placed in lateral dry air flow or lyophilized (Jaronski, unpublished data). Cold saline solution was necessary to keep the conidia from germinating, and the filter aid allowed the creation of a fine friable powder at the end of the process.

11.2.6.1.6 Major technical problems/solutions in solid substrate fermentation

The greatest problem in solid substrate fermentation is the scale-up to large capacity at commercial levels. For a mycoinsecticide to succeed commercially, a very large number of conidia must be produced and produced as cheaply and efficiently as possible to compete with chemical insecticides. There are two directions in the production of entomopathogenic fungi by solid substrate fermentation for inundative applications, whether it be uni- or biphasic. One is a low input, manually intensive, simple technology, like plastic bags. In practice, especially in emerging rural economies, this system is appropriate and often targets limited local use. The other is a high technology, high input, industrial approach, as can be seen in companies in North America and the EU. The industrial approach is mandated by the considerable costs of development and registration, and expensive wage rates. The typical bag fermentation is not amenable to such large-scale needs, particularly in the developed world where salaries are considerable. The high technology alternative is capital intensive, often requiring specialized equipment (Ravensberger, 2011), although sparing manpower costs.

Applied to large-scale solid substrate fermentation, the need for large conidial numbers mandates efficient sterilization of very large amounts of a solid substrate. For example, for one US company the fermentation batch size is 10,000 kg. Traditionally, moist heat (steam) is the method used. The V-cone blender sterilizer mentioned in Section 11.2.6.1.3 is one solution. There are alternative methods, for example, radiation sterilization, already in commercial use in other industries.

They may be adaptable to fungal mass production. Of course, with plastic bags, the unit volumes are small (0.1−2 kg) and so relatively easy to steam sterilize. The use of recyclable solid substrate is highly desirable, yet the current state of the art largely relies on a grain, causing considerable waste disposal issues. In countries with emerging economies, many different alternative materials have been identified, but few seem to have been adopted by commercial enterprises.

Jenkins (1995), in reviewing yields of conidia for 13 fungi produced on the solid substrate at that time, found only one instance where yields reached 1×10^{13} conidia kg dry substrate. The product, based on *B. bassiana*, which is manufactured by CertisBio USA (originally Mycotech Inc., then Emerald BioAgriculture, later Laverlam International) was reported to have an operational yield of 2.6×10^{13} conidia/kg substrate (Bradley et al., 1992).

There is also the demand for sufficient, yet efficient, fermentation space, although the mushroom and soy sauce industries have dealt with such problems and offer potentially useful technology. Harvesting problems are largely solved with the advent of cyclone dust collector technology as discussed in Section 11.2.6.1.5.

11.2.6.2 Submerged fermentation

Submerged liquid fermentation to produce mycoinsecticides has been a goal of fermentation microbiologists for many years. The technology lends itself to massive scale-up, using existing commercial equipment, and allows closer control of environmental variables, and shorter process times, that is, hours rather than days as for solid substrate fermentation. Submerged fermentation is currently used to produce commercial mycoinsecticides of *C. fumosorosea* (Kepler, B. Shrestha & Spatafora) and *Akanthomyces* spp. In the past 7−8 years methodology for blastospore production has undergone significant advances, particularly for *B. bassiana* and *C. fumosorosea* as well (Mascarin et al., 2015a, 2015b, 2016, 2018). These advances potentially open the way for realistic commercial production of these fungi by submerged fermentation for blastospores as the active ingredient and are discussed in more detail in Sections 11.2.6.2.2 and 2.6.2.3.

11.2.6.2.1 The end products of submerged fermentation

Submerged fermentation generally yields different propagules than solid substrate fermentation: blastospores, submerged, "microcycle" conidia, stabilized mycelial products, and microsclerotia. Blastospores (Fig. 11.8A and C) are vegetative cells by which the Ascomycetes proliferate through the body of an infected insect (Vestergaard et al., 1999). Blastopore multiplication is yeast-like, but not the true budding of yeasts. Most or all species of *Beauveria*, *Metarhizium*, and *Cordyceps* will grow as blastospores under the appropriate liquid fermentation conditions. The blastospore is a more environmentally fragile propagule than the aerial conidium and special approaches are necessary to make them desiccation-tolerant.

Submerged, or microcycle, conidia are produced by species within the genera *Beauveria* and *Metarhizium* (Fig. 11.8B and D) (Thomas et al., 1987; Bosch and Yantorno 1999; Zhang, 2001). Microcycle conidiation has been defined as the production of conidia directly by a spore without the intervention of hyphal growth (Anderson and Smith, 1971). These conidia are

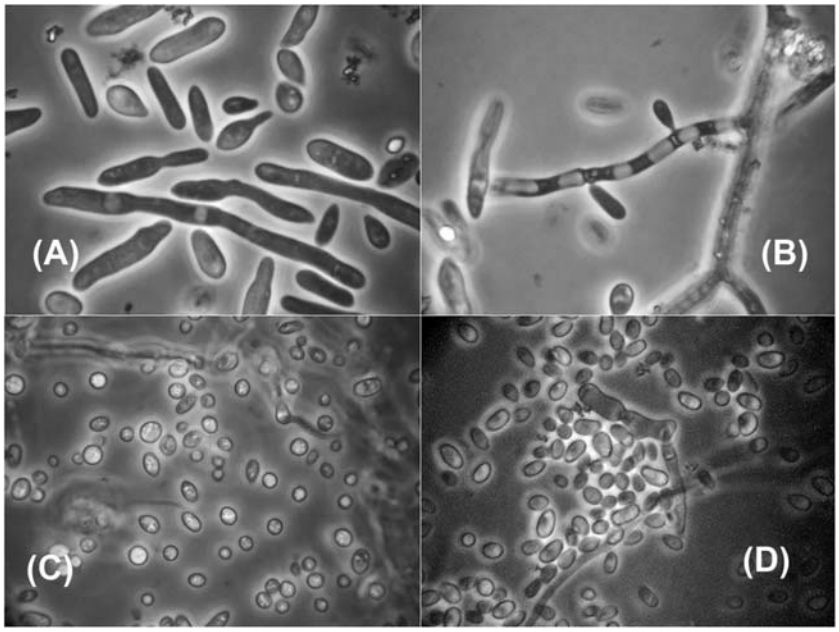

FIGURE 11.8 Ascomycete propagules obtained in submerged liquid fermentation: (A) blastospores of *Metarhizium anisopliae*; (B) hyphae of *Metarhizium robertsii* giving rise to microcycle conidia; (C) blastospores of *Beauveria bassiana*; (D) microcycle conidia of *Metarhizium acridum*.

morphologically and ultrastructurally different from true, aerial conidia; they lack one layer in the spore wall and have some different physical properties (Hegedus et al., 1990). They also germinate at a rate intermediate between blastospores and aerial conidia. The microcycle conidia of *Metarhizium acridum* (Driver and Milner) can evidently also be produced on a solid medium (Zhang et al., 2009). Since 2010 production of microcycle conidia seems to have been generally ignored.

Submerged fermentation can be used to produce mycelial masses, which are then dried with preservatives to make granular formulations, such as was described in Section 11.2.4 for the Entomophthorales (Rombach et al., 1988). When rehydrated, the mycelium generates large numbers of conidia. For a time during the 1990s, a *Metarhizium*-based, dried mycelial pellet, which would conidiate when rehydrated upon application to soil, was commercialized by Bayer AG, as BIO1020. These pellets were produced in submerged fermentation using specific media and additives, which caused the mycelium to aggregate as large pellets. The pellets could then be harvested and dried to form 0.5 to 1 mm diameter granules (Andersch et al., 1993). This product and process were subsequently abandoned.

Fungi within the genus *Metarhizium* can also produce compact melanized bodies, 50−200 μ in size, termed microsclerotia, because of their similarity to such forms in plant pathogenic fungi (Fig. 11.9A). These microsclerotia are generated under certain nutrient and aeration conditions (Jackson and Jaronski, 2009; Jackson and Jaronski, 2021; Mascarin et al., 2014; Paixão et al., 2021). The process has been scaled up to 100-L bioreactors and appropriate harvesting methods developed to provide at least 1 year's shelf life of properly dried preparations (Jackson and Jaronski, 2012). So far, all species within the genus *Metarhizium* tested seem to produce microsclerotia, although the numbers produced seem to vary by strain (Jackson and Jaronski, 2009). Experiments with multiple strains of *B. bassiana* failed to yield microsclerotia using the media and fermentation conditions for *Metarhizium* (Jackson and Jaronski, unpublished data). Very recently, the production of microsclerotia, or microsclerotia-like bodies, by strains of *B. pseudobassiana* and *B. brongniartii* has been reported (Jing-Ling et al., 2011; Yang et al., 2014; Villamizar et al., 2018; Huarte-Bonnet et al., 2019; Villamizar et al., 2021). In each case, different media from those required by Jackson and Jaronski (2009) for *Metarhizium* were used (see Section 11.2.6.2.2). In 2014 *M. rileyi* was added to the fungi able to produce microsclerotia using modifications of the Jackson Jaronski medium (Song et al., 2014). This inclusion is not surprising because the species has recently been reassigned to the genus *Metarhizium*. Two strains of *A. lecanii* were reported to produce microsclerotia (Wang et al., 2013). These results need to be verified with additional strains of this species.

Microsclerotia can be simply harvested by filtration with or without a filter aid (diatomaceous earth, attapulgite clay) and air-dried. When microsclerotia are rehydrated, they rapidly conidiate and can thus be made into conidiogenic granules for use (Fig. 11.9B). A noted feature is that microsclerotia can be simply air-dried, have considerable shelf-life stability, and the resulting granules can be sized as needed for particular applications. There are a few considerations, however, when choosing the most appropriate propagule for deployment against an insect. The reader is referred to Jackson et al. (2010) for an exploration of this topic; the discussion is not restricted to the genera *Beauveria* and *Metarhizium*, but also includes relevant data about *C. fumosorosea*, which is otherwise discussed in Section 11.2.6.4.1.

11.2.6.2.2 Media

Blastospore production has long been a target for creating a commercially viable propagule. Initial research (Adamek 1965) identified a liquid medium for *M. anisopliae* blastospore production: 30 g corn steep liquor/L, 40 g glucose/L, 40 g yeast extract/L, and 4 mL sorbitan monooleate (Tween 80®)/L. In retrospect, the increased osmolarity due to the

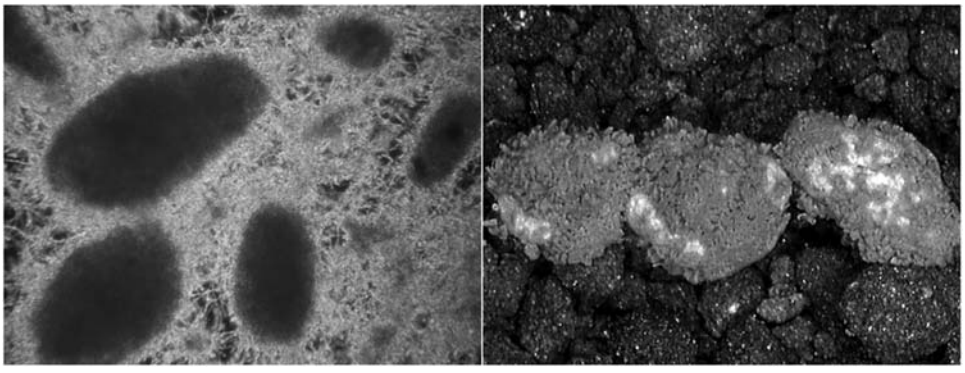

FIGURE 11.9 (Left) Microsclerotia of *Metarhizium brunneum*; (Right) granules formed from microsclerotia that have conidiated upon rehydration on moist soil.

glucose and monosorbitan oleate was required for blastospore production. Studies by others in subsequent years concerned media optimization. A modified Adamek medium emerged as the most productive. It features an elevated very high concentration of glucose (140 g/L) and corn steep liquor (80 g/L) (Iwanicki et al., 2018; Iwanicki et al., 2020; Iwanicki et al., 2021). Elevated osmotic pressure from the high glucose concentration is critical for the shift from mycelial to blastospore production, as is a high aeration rate (Iwanicki et al., 2018). The yields were $4-6 \times 10^8$ blastospores/mL within 2 days of fermentation, but these levels required a preculture of a different modification of Adamek's medium, having only 40 g glucose/L, which was then used to inoculate the main fermentation to an initial level of 5×10^6/mL. There was considerable variability among the 11 *Metarhizium* strains (spanning several species of this fungus) in their response to this medium (Iwanicki et al., 2018). In my own laboratory, *M. brunneum* F52 tends to the mycelial formation and fails to produce many blastospores in their medium, while *M. robertsii* DWR2009 reaches the levels reported by Iwanicki et al. (2018) (Jaronski unpublished data).

Research into blastospore production by *Beauveria* spp. dates from the late 1980s (e.g., Humphreys et al., 1989). However, submerged fermentation for *B. bassiana* was not commercially pursued because of commercially unacceptable low yields, excessive "contamination" with mycelium preventing the creation of a readily sprayable formulation, low blastospore tolerance to the desiccation needed for wettable powder formulations, and poor shelf life, especially under nonrefrigerated conditions. Research by Mascarin et al. (2015a), Mascarin et al. (2015a, 2015b, 2018) has greatly expanded the potential utility of *Beauveria* spp. as well as *Cordyceps* spp. blastospores, and this work merits special attention here. Key to high yields of *B. bassiana* blastospores is a very high concentration of glucose, 100−140 g/L, a commensurate amount of casein or cottonseed flour nitrogen source to achieve a C:N ratio of 21:1 or 23:1 (25−30 g of either substance/L), and considerable aeration during fermentation. However, the responses of different *Beauveria* spp. strains to such media can vary (Mascarin et al., 2018) as with *Metarhizium* spp. (Iwanicki et al., 2018). Five strains of *B. bassiana* demonstrated varied blastospore yields after 3 days of fermentation, from 1.2×10^8 to 1.24×10^9 blastospores/mL. Similarly, five strains of *C. fumosorosea* produced from 6.9×10^8 to 2.79×10^9 blastospores/mL. Cottonseed meal, in particular, was identified as a very satisfactory nitrogen source, better than hydrolyzed casein (Mascarin et al., 2018).

The production of microcycle conidia by *B. bassiana* was first described by Thomas et al. (1987). Their medium consisted of 5% glucose and 1% KNO_3. This medium preferentially produced high numbers of submerged conidia while other carbon sources strongly favored blastospore production. In work refining the production of *B. bassiana* blastospores and microcycle conidia, Chong-Rodríguez et al. (2011) discovered that a medium of glucose plus casamino acids or one containing glucose, peptone, and KNO_3 produced high numbers of blastospores, while a glucose-KNO_3-corn steep liquor medium produced mostly microcycle conidia. Slight variations in the ratio of carbon and nitrogen, or the nature of the carbon source, will also affect blastospore yields. For example, with 5% sucrose as the carbon source, *B. bassiana* produced 4.6×10^8 blastospores and microcycle conidia/mL with a 9:1 ratio of the two. When the media was supplemented with 5% sugar beet molasses *B. bassiana* generated 2×10^9 spores/mL but only 64% were microcycle conidia with the remainder being blastospores (Jakobs-Schönwandt et al., 2011). Strain and species differences increase the complexity in defining an optimal medium for either blastospores or microcycle conidia (Mascarin et al., 2015b, 2018; Vega et al., 2003). In addition, the production of microcycle conidia proceeds in parallel with that of blastospores and hyphal fragments, "contaminating" the microcycle conidia. This situation creates problems in creating a microcycle conidium pure preparation for formulation.

For *M. acridum*, organic nitrogen, preferably as brewer's yeast, and high levels of sucrose seem to be essential for "submerged conidia" production (Jenkins and Prior, 1993; Leland et al., 2005a). This fungus will produce abundant blastospores in a medium of glucose and C:N ratio > 30:1, using casamino acids as the nitrogen source (Jaronski, unpublished data). Issaly et al. (2005) further refined medium and fermentation parameters for *M. acridum*, affirming the use of sucrose and yeast extract and identifying an optimal C/N ratio of 1:6 for blastospores. Zhang et al. (2009) described the production of microcycle conidia by one strain of *M. acridum* on an agar medium consisting of 3% sucrose, 0.5% yeast extract, 0.3% $NaNO_3$, and trace salts. The same fungus conidiated normally on quarter-strength Sabouraud dextrose agar (1% dextrose, 0.25% neopeptone, 0.025% yeast extract). Whether other *M. acridum* strains do the same remains to be determined. A high osmolarity medium (using polyethylene glycol) seems to have allowed the formation of a spore intermediate between blastospores and microcycle conidia in morphology and physical−-chemical properties. These spores germinated more quickly than submerged conidia at a rate similar to blastospores and were more pathogenic than either submerged conidia or aerial conidia (Leland et al., 2005b). Fargues et al. (2002) added 0.4% polyoxyethylene sorbitan monooleate (Tween 80®) to increase submerged spore production.

For the production of marcescent mycelium, similar methods as for the Entomophthorales have been employed (Rombach et al., 1988). Magalhães et al. (1994) identified 4% sucrose and 1% yeast extract as the best medium of

several assessed for an isolate of *M. anisopliae*, yielding 9 g biomass/L in 72 hours and 1.6×10^{10} conidia/g dry mycelium after rehydration. This process has had very little implementation, however, and is best suited for small scale, artisanal applications. The Bayer mycelial pellet mentioned in Section 11.2.6.2.1, was produced in a glucose-yeast autolysate-K_2HPO_4 medium with $MgCl_2$ and trace salts. The medium was seeded at a very low rate (10^6 conidia/mL for small volumes, or if a preculture was used, then it was added at 3% of final volume) to induce pellet formation (Andersch et al., 1993).

Production of *Metarhizium* spp. microsclerotia requires a high concentration of glucose, for example, 32 g/L, and a C:N ratio in excess of 30:1, as well as trace salts (Jackson and Jaronski, 2009, 2021); vitamin supplementation as described in their 2009 paper may not be necessary but the salts are required (Jaronski, unpublished data). A patent in the US as well as for several other countries for their process and for use of microsclerotia has been granted (Jackson and Jaronski, 2021). This patent has been licensed by a multinational company and is presumably being scaled up to a commercial level (Jaronski, unpublished). For *M. rileyi*, then called *Nomuraea rileyi*, Song et al. (2014) initially used a medium consisting of 32.0 g glucose, 2.0 g ammonium citrate, and 0.15 g $FeSO_4$/L, later optimizing and scaling fermentation to 20 L (Song et al., 2017). In the course of their optimization, they observed that very high rates of aeration were necessary to achieve the greatest yields (3.6×10^4 microsclerotia/mL). In contrast, microsclerotial production by the *B. pseudobassiana* and *B. brongniartti* strains, reported by Villamizar et al. (2018), required a different medium: 15 g casamino acids/L, and 75 g corn steep liquor/L plus salts, as well as the usual trace salts and vitamin supplements reported by others. Huarte-Bonnet et al. (2019) used 10 g glucose/L and 5 g yeast extract/L plus trace salts for their *B. bassiana* strain; the yield of microsclerotia was very low, $2-9 \times 10^3$/mL, however, several orders of magnitude less than that reported by Villamizar et al. (2018). A key factor for microsclerotia production by *B. bassiana* seems to be inclusion of 0.2 g/L of Fe^{+2} as $FeSO_4-7H_2O$ (Trejo et al., 2020). US patent protection is being sought for the use of Fe^{+2} (Trejo et al., 2020). However, I must add that I failed to produce microsclerotia of *B. bassiana* strains GHA, ANT03 and PPRI5339 in numerous attempts using the media of both Villamizar et al. (2018) and Huarte-Bonnet et al. (2019), even with the $FeSO_4$ included (Jaronski, unpublished data). Whether this discrepancy is due to different *Beauveria* species/strains being used, or some other factor(s), remains to be resolved. A medium of 50−72 g glucose/L and 28−50 g soybean powder/L (for C:N ratio >5:1) caused two strains of *A. lecanii* to produce considerable number of presumed microsclerotia (Wang et al., 2013).

11.2.6.2.3 Fermentation parameters (air, agitation, pH)

A high degree of aeration in submerged fermentation is essential. The difficulty lies in the method of aeration. On a small, flask-scale level, rotary agitation at 300−400 rpm., and ideally with baffled flasks should be used. For *C. fumosorosea*, 20% dissolved oxygen is necessary for good blastospore production (Jackson, 2012). Machado et al. (1994) stated that the best air flow rate was 1.5 volumes of air per volume of medium per minute for mycelium production by *M. anisopliae*. De la Torre and Cardenas-Cota (1996) used air flow and agitation to achieve 20% saturation for optimal production of *C. fumosorosea*. A very high oxygenation rate was required with *B. bassiana* (Mascarin et al., 2015a). High aeration is a factor of concern in expanding fermentation to hundreds, thousands of liters. The traditional stirring paddle method of agitation in larger volume fermenters can lead to mycelium and blastospore damage through the shear forces present at required speeds. Sparging fermenters overcome this problem but may not provide sufficient oxygen efficiently in larger volumes.

While initial pH has been adjusted in many of the published studies concerning submerged fermentation, it is typically changed during the fermentation by the fungus, and often left uncontrolled during the fermentation without effect.

Recommended temperatures are those at which the particular fungus grows best, typically 24°C−28°C although there is one report that heat induction of C. *fumosorosea* inoculant for submerged fermentation strongly increased microcycle conidiation (De la Torre and Cardenas-Cota, 1996). In their study, they exposed the inoculum, a conidial suspension, to an initial 24-hour incubation at 37°C followed by 96 hours at 30°C. The germinating conidia immediately went into blastospore rather than mycelial growth and quickly gave rise to microcycle conidia. Anderson et al. (1978) saw a parallel heat induction of microcycle conidiation in *Paecilomyces varioti* Samson. Whether this phenomenon extends to *Beauveria* spp. or *Metarhizium* spp. remains to be determined.

11.2.6.2.4 Equipment for submerged fermentation

Discussion of equipment used in submerged fermentation especially in large scale, industrial production is beyond the scope of this review. Typically, the same fermentation equipment as is used for other microorganisms is used for the

entomopathogenic Ascomycetes. The literature about submerged liquid fermentation to produce microorganisms is vast and the reader is referred elsewhere for more information (e.g., Stanbury and Whitaker, 1993; Rao, 2010).

11.2.6.2.5 Downstream processing

The product of liquid culture is typically harvested by filtration and then processed further to stabilize the fungal material. Industrial-scale filtration uses a ribbon filter or continuous flow centrifugation. Often diatomaceous earth or attapulgite clay is added to aid in filtration because fungal culture will often clog filters.

If the fungus material (blastospores, microcycle conidia) is to be used within a short time after harvest, it can be preserved by refrigeration. Otherwise, proper preservation of the propagules is necessary for acceptable shelf life and optimal handling characteristics in formulations. Conversion of the fungal propagules into a dry powder can be accomplished by simple air drying, freeze-drying, or spray drying, but the preservation of the propagules, especially blastospore, viability has been the greatest challenge. Historically, most of the research regarding blastospore preservation was directed toward *C. fumosorosea*, rather than *Beauveria* spp. or *Metarhizium spp*., until recently. Typically, blastospores lose considerable viability upon drying (Inch et al., 1986; Fargues et al., 1994). Microcycle conidia are less prone to this phenomenon but do suffer viability loss.

In simple air drying, the relative humidity of the drying air can significantly affect the desiccation tolerance of *C. fumosorosea* blastospores (Jackson and Payne 2007). A humidity >40% allowed significantly higher rates of initial blastospore survival after drying versus drying with lower humidity air; air with an RH >50% improved the shelf-life of the air-dried blastospore preparations. Mascarin et al. (2015a) used lateral air inflow and controlled humidity (50%−60% for the initial 15−19 hours and the 15%−25% for the final 1 hour), achieving a final moisture content of less than 4% (water activity ~ 0.35), to obtain initial blastospore viability of 61% for *B. bassiana* and 70%−86% % for *C. fumosorosea* with strain-dependent differences. Fargues et al. (1979) discovered good blastospore preservation by lyophilization using powdered milk supplemented with glycerol as cryoprotectants. Lyophilization, however, is not economically practical on a large scale in many situations. Spray drying, however, is a common technique used in many microbial fermentations. Stephan and Zimmermann (1998) obtained 90% blastospore/microcycle conidium viability after spray drying *B. bassiana*, *M. anisopliae*, *M. acridum*, and *C. fumosorosea* when the blastospores were suspended in 20% skimmed milk powder and 2.5% sugar-beet syrup before spray drying; unprotected submerged spores were killed. Yeast extract, soluble starch, hydroxyethyl-starch and bentonite clay failed to protect the propagules. Spray dryer inlet and outlet temperature, and the flow rate, are also important to minimize damage to blastospores. Stephan and Zimmermann (1998) documented that 64°C and 48°C were the respective critical temperatures for inlet and outlet temperatures. Of relevance to this discussion is the work of Horaczek and Viernstein (2004) who reported a detailed comparison of lyophilization, spray-drying, and fluid-bed drying for the processing of aerial conidia of *Beauveria brongniartii* (Saccardo) and *M. anisopliae*, harvested with water from agar substrate. They found that (1) *M. anisopliae* was slightly more heat resistant than *B. brongniartii* (2 minutes at 50°C); (2) conidia of both fungi suffered considerable mortality (~65%) after spray drying, even with a 60°/40°C inlet/outlet temperatures; (3) *B. brongniartii* was best preserved with lyophilization, whereas the drying phase caused considerable mortality to *M. anisopliae;* (4) *M. anisopliae* was better preserved with either of two possible preservatives, skim milk and polyvinylpyrrolidone; (5) fluid bed drying killed both fungi even when the inlet temperature was lowered to 60°C Chen and Feng (2002), in examining several drying methods for *C. fumosorosea* conidia, observed that low vacuum (0.1 MPa), low heat (30°C) drying for 20−24 hours was the best, yielding only slight loss in conidial viability. High vacuum, freeze-drying, high vacuum room-temperature drying, and heating-drying at 35°C were all deleterious for conidial viability. Thus, desiccation and temperature intolerance are the most important constraints in processing conidia, and likely even more so for blastospores and microcycle conidia.

In the last few years, there have been considerable advances in understanding how to preserve blastospore viability during drying, notably work by Mascarin et al. (2016, 2018, 2021) for *Beauveria* spp., and Iwanicki et al. (2018, 2021) for *Metarhizium* spp. Fairly rapid air-drying seems to be superior to spray drying even with a protectant (Mascarin et al., 2016). Spray drying with skimmed milk powder resulted in approximately 17% loss in initial viability, but the shelf-life of these blastospores was very poor at 28°C, dropping to 0% by 6 weeks At 4°C blastospore viability seemed to stabilize at approximately 60% absolute viability out to 39 weeks, when the study was terminated (Mascarin et al., 2016). On the other hand, air drying with diatomaceous earth caused only a 20% drop in viability during drying, with a subsequent half-life of 13 weeks at 28°C decreasing to 0% viability at 9 weeks. At 4°C, however, these air-dried blastospores maintained close to initial viability at 39 weeks, when the study was terminated. All these studies were conducted with vacuum packing and dried blastospore preparations. When a combination oxygen scavenger-desiccant (RP-

3A®, Mitsubishi Gas Chemical America) was added to the air-dried blastospores, shelf life at 28°C was greatly improved, with only a small viability loss during 1 year. This performance is commercially acceptable. Poor preservation of blastospore viability occurred when only iron-based oxygen scavengers or silica desiccant were used by themselves. Good preservation of blastospore viability required both components. Lastly, Iwanicki et al. (2021) indicated that the shelf life of dried *M. anisopliae* blastopore preparations, when produced in their glucose:corn steep liquid medium, was optimized by similar vacuum packaging with an oxygen/moisture scavenger and cold storage.

An effort has also been concentrated on media manipulations to produce more desiccation-tolerant blastospores. For example, Cliquet and Jackson (1999) evaluated the impact of amino acids, carbohydrates, trace metals and vitamins on the freeze-drying tolerance of *C. fumosorosea* blastospores as well as hyphal growth and sporulation. Sodium citrate or galactose as the sole carbohydrate produced more-desiccation-tolerant spores but yielded lower blastospore concentrations. Media containing glucose concentrations greater than 20 g/L and 13.2−40 g casamino acid/L supported maximal production of desiccation-tolerant blastospores of *C. fumosorosea* using air drying but not necessarily the greatest yields per se (Jackson et al., 1997). Thus, there seems to be a tradeoff between blastospore yield and desiccation tolerance. In the case of the BIO1020 mycelial pellets, they were simply harvested using a 0.1 mm pore sieve plate, washed and dried in a fluidized bed granulator (Andersch et al., 1993) or freeze-dried. Mascarin et al. (2015b) noted that the use of cottonseed flour with a very high concentration of glucose to produce *B. bassiana* blastospores enhanced desiccation resistance on their drying. On the other hand, casein hydrolysate as the nitrogen source was associated with slightly greater desiccation tolerance in *C. fumosorosea*, again with strain-dependent differences. In a further study, two strains of *B. bassiana* demonstrated different responses to particular nitrogen sources not in initial survival, which was 64%−79%, after drying, but in subsequent shelf life at 4°C (Mascarin et al., 2018). They hypothesized "An important feature of nitrogen compounds concerns the content of soluble amino N and the total amount of free amino acids, which seems to influence blastospore tolerance to dehydration." Nevertheless, shelf life under refrigeration was very poor, with viability after 9 months being a maximum of 55% for one strain and 24% for the other.

Microsclerotia can be easily harvested by filtration, facilitated by the addition of 3%−5% W:V diatomaceous earth or attapulgite clay (Jackson and Jaronski, 2009, 2012), granulated, and air-dried. Good viability of the microsclerotia was obtained when the crumbled filter cake was air-dried for 2 days at 30°C, with the lateral inflow of air having 50% RH (Mascarin et al., 2015a). A critical aspect is that the microsclerotia granules must be dried to below water activity of 0.3 (∼2.7% moisture). Filtration can be easily automated and throughput increased for hundreds of liters of fungal slurry (Jackson and Jaronski, 2012). Jackson and Jaronski (2009, 2012) observed that the best shelf life of very dry microsclerotia granules was in vacuum packaging of the dry formulation. Disruption of the vacuum environment caused the microsclerotia to lose viability in a few months even at 5°C, with the dry state being maintained (Jaronski, unpublished data).

11.2.6.2.6 Major technical problems/solutions in submerged fermentation

There are several major problems with blastospore or microcycle conidia production using submerged fermentation. Rarely are the desired propagule produced in high purity. Blastospores are "contaminated" with mycelium, while blastospores and mycelium are co-present with microcycle conidia. For example, a mixture of cell types dilutes out the desired type and necessitates larger fermentation volumes for a specified number of the desired spores. Such mixtures make harvesting and purification difficult; or render the physical nature of the harvested and stabilized product incompatible with formulation and satisfactory spray characteristics. Filtration, for example, through 80-mesh (165 μ) sieve, to remove hyphae and mycelia may be necessary, as was used by Mascarin et al. (2016) in their study. Certainly, most hydraulic spray nozzles and in-line filters require all particles to pass through 80-mesh screen. Ultra-low volume sprayers and spinning disk sprayers require even smaller particles. The hydrophilic nature of blastospores and microcycle conidia requires different liquid formulations than do the hydrophobic aerial conidia. Also, with few exceptions, the shelf life of dry blastospore or microcycle preparations is shorter than that of dry aerial conidia, oftentimes commercially unacceptable, without special measures, such as vacuum packaging, low-temperature storage, or some sort of encapsulation. All of these measures can add considerable cost to a commercial mycoinsecticide, limiting user acceptability. Microsclerotia production with *Metarhizium* spp. or *Beauveria* spp., on the other hand, offers a viable alternative, where sporulating granules (in soil, under a plant canopy, or in other humid, protected microhabitats) are practical. Microsclerotia seem to be considered stable as a granular formulation, especially when vacuum packaged, and produce abundant conidia when rehydrated.

11.2.6.3 Other, novel, production methods

In addition to several atypical methods for fungus production described by Bartlett and Jaronski (1988), a novel technology using media-impregnated nonwoven fabric bands was developed and commercialized in Japan in the mid-1990s

as "Biolisa kamakiri" utilizing *B. brongniartii* (Higuchi et al., 1997; Fujiwara-Tsujii and Yasui, 2021). A US patent subsequently described the methodology and specifics for mechanization and scale-up (Higuchi et al., 1996).

Wood pulp fabric, and in later modifications rayon-polypropylene or rayon polyester, typically 0.5−2 mm in thickness and sometimes laminated, is first impregnated with a liquid culture medium, then is heat dried, with the drying process sterilizing and stabilizing the media. This fabric can be stored dry. The patent describes a machine that can produce the dry, medium-impregnated fabric in long rolls. A hydrophilic polymer added to the fabric improves medium absorption and fungal conidiation. To cultivate a fungus for conidial production, the fungus is first grown in a liquid medium, which is then applied to the dry fabric. With suitable humid incubation ($>80\%$ RH, $\sim 25^{\circ}$C) for 3−14 days, the fungus abundantly colonizes and conidiates on the surface and even within the fabric, which is then partially or completely dried and stored refrigerated until use. Levels of $1-2 \times 10^8$ conidia/cm^{-1} were obtained in this manner. The method was developed for the deployment of *B. brongniartii* against tree-dwelling Chrysomelidae beetles, who have a habit of moving up and down tree trunks. A conidiated band is wrapped around the tree trunk, and the insects pass beneath or over it in their movements, coming into contact with the conidia. If the semidry band is rewetted, for instance by rain, there is another flush of conidiation. This fabric carrier was also used to obtain *Lecanicillium* conidia at similar levels (Higuchi et al., 1996). US workers have adapted the fabric methodology for a *M. brunneum* in an effort to evaluate its potential to combat the Asian Longhorned Beetle [*Anoplophora glabripennis* (Motschulsky)] (Dubois et al., 2004; Hajek et al., 2006; Shanley et al., 2009). The approach was also evaluated and showed promise vs. pecan weevil (Shapiro-Ilan et al., 2009).

In parallel to the above development, Jenkins and Prior (1993) developed a similar method for mass production of *M. acridum* conidia on cellulose cloths. In their case, however, the strategy was to remove the conidia of the cloths by washing or by mechanical separation and Sir classification. The method was abandoned in favor of grain-based solid substrate fermentation.

Bringing the liquid fermentation facility to the side of the field or in the form of a self-contained, portable fermenter was another novel concept developed in the 2000s (Jackson et al., 2004). By the late 1990s production of beneficial bacteria on site for use as soil amendments in managed turf had been demonstrated and that technology was adapted to entomopathogenic fungi. Jackson and his associates developed and validated a self-contained system for producing *C. fumosorosea* blastospores on-site for immediate application. Prepared media concentrates were diluted in a chemically disinfected (but not completely sterilized), portable, 100 L fermenter that could be placed beside the field or glasshouse intended for application. A rehydrated, dry blastospore preparation was used as inoculum. Fermentation variables were monitored and controlled by a computer attached to the unit. Yields of 8×10^{11}/L blastospores were obtained after 48 hours of fermentation starting with an inoculum level of 5×10^9 blastospores/L medium. Periodic bacterial contamination was a periodic problem, however, despite the use of low pH (4.0). The system was designed to accommodate limited user expertise and the greatest self-containment possible. This technology is readily adaptable to *Beauveria* spp., *Metarhizium* spp., and the other Ascomycetes. Commercial development ensued, initially targeting high-value turf situations but was eventually ended due to cost. There have been no recent developments in this regard.

11.2.6.4 Other ascomycetes

11.2.6.4.1 Cordyceps (Isaria)

The Ascomycetes *C. fumosorosea* and *C. javanica* have generated considerable interest and commercial exploitation for the control of several glasshouse pests in recent years. De Faria and Wraight (2007) identified seven commercial products containing putative *C. fumosorosea* at that time; in the US and EU, there are currently two registered strains, Apopka97 and FE9901. Recently, however, the nomenclature has been muddied by the discovery that some strains of *C. fumosorosea* are actually *C. javanica*, including Apopka 97 (Ayala-Zermeño et al., 2015; Gallou et al., 2016). Whether or not these nomenclatural assignments of strains are paralleled by differences in media, etc., remains to be determined. A second species, *I. farinosa* (Holmsk.) Fr., has been occasionally identified as an insect pathogen of potential use, but there has been little effort directed toward its mass production.

While *C. fumosorosea and C. javanica* can be produced using solid substrate fermentation, using substrates and methods as developed for *Beauveria* spp. and *Metarhizium* spp., many strains often have a far blue-near UV light requirement for good conidiation (Sakamoto et al., 1985; Sanchez-Murillo et al., 2004; Kuźniar, 2011). In continuous darkness, conidiation seems to be reduced to continued, robust, vegetative growth. This situation may be the cause of the relatively low yields in at least one assessment of different grains for producing *C. fumosorosea* conidia (Kuźniar and Krysa, 2011). A need for light:dark cycles may also be present in some *C. fumosorosea* strains (De la Torre and Cardenas-Cota, 1996). However, not all strains of *I. fumosoroseus* display this behavior. In screening many isolates of

this species for potential commercialization, Mycotech Corporation identified 4−5 isolates that conidiated abundantly on agar or a solid substrate in continuous darkness (Jaronski, unpublished data).

Current commercial production seems to be based on liquid fermentation. Eyal et al. (1994) describe liquid fermentation production of *C. fumosorosea* Apopka 97 using molasses, cotton seed flour and corn steep liquor to produce blastospores and mycelium that are then encapsulated in an alginate matrix to yield prill having the potential of producing conidia upon rehydration. This system formed the basis of commercial production of PFR97®; current processes are proprietary. FE9901 is produced by its company in submerged fermentation and consists of blastospores. Considerations in producing *Isaria* blastospores by submerged fermentation are discussed in Section 11.2.6.2

11.2.6.4.2 Akanthomyces (Lecanicillium)

Lecanicillium muscarium Zare and Gams and *L. longisporum* Zare and Gams (both formerly classified as *Verticillium lecanii* (Zimmerman)) have attracted some attention as biocontrol agents of Homoptera and spider mites. In their 2007 survey, De Faria and Wraight (2007) noted 16 commercial products in existence. One strain, Ve6, has been in commercial use in Europe since the 1980s. This genus is notable in that conidia are borne in slime balls, rarely in dry chains, unlike the other Hyprocreales fungi, which produce hydrophobic conidia.

Both submerged liquid and solid substrate fermentation have been used to produce these two fungi, using methods similar to those for the genera *Beauveria* and *Metarhizium*. Both aerial conidia and submerged conidia are produced in the respective fermentation systems. There are differences in morphology, germination and growth patterns between the two types, but there is no clear indication in the literature about their relative desiccation tolerance.

The production process used for the commercial *A. muscarium* Ve6 in the EU is proprietary but is possibly submerged liquid fermentation. Derakhshan et al. (2008) reported that molasses-yeast broth was the best liquid medium while rice yielded the highest conidial production. A wheat bran-sugarbeet pulp mixture (9:1 w/w) has also been touted as an excellent solid substrate (Grajek, 1994). Feng et al. (2000) identified rice bran to be the best medium, better than cooked rice, with bran:husk (1:1) was almost as good. Of note is that spore production in liquid media, at least shake flask culture, decreased significantly after 9 days, indicating a dynamic situation in that system. Several agricultural waste products have also been examined with a view to lowering production costs (Lopez-Llorca and Carbonell 1998; Lopez-Llorca et al., 1999; Shi et al., 2009). The suitability of sorghum grain, rice grain, farm manure, vegetable waste, vermicompost, sugarcane bagasse, and neem seed kernel were examined with the first two materials, supplemented with 1% yeast extract, superior to the others (Kumar et al., 2020). Their data are, however, difficult to understand. These media had to be soaked overnight in water, and then cooked, before autoclaving, a process that does not lend itself to anything more than artisanal production.

Optimal temperatures for growth and sporulation in both systems are typically 20°C−25°C. There is virtually no public information about the harvesting and subsequent processing of *Akanthomyces*. Presumably, the aerial conidia can be washed off solid substrate because they are not hydrophobic; submerged conidia can be harvested by methods described for other Hypocreales.

Akanthomyces lecanii can evidently produce microsclerotia in liquid fermentation (Wang et al., 2013). The reader is referred to Section 11.2.6.2 for more information.

11.2.6.4.3 Hirsutella thompsonii

Hirsutella thompsonii Fisher has undergone several commercialization efforts, primarily in the US in the 1970s, and in Cuba in the 1980s. Only one product made from this fungus was identified by De Faria and Wraight (2007) in their survey. A more recent review of this genus is provided by Reddy et al. (2020), but the authors present very little information about mass production.

Initial data (VanWinkelhof, McCoy, 1984) indicated that microcycle conidia could be produced in submerged fermentation, but by only 1 of 15 strains evaluated and conidial germination was poor. The earliest program used liquid fermentation to produce mycelial mats, which were refrigerated and then formulated on the day of application by creating an aqueous slurry for spraying (McCoy et al., 1975). The medium consisted of dextrose, yeast extract, peptone and essential mineral salts. Subsequently, solid substrate fermentation, using wheat bran, was substituted by the company commercializing the fungus (Jaronski, unpublished data). The fungus was marketed for the control of eriophyid mites in citrus, but sales were terminated in the 1980s for a number of technical reasons. Latge et al. (1988) were also able to obtain microcycle conidia from submerged fermentation but from a strain unique in this regard. There have been no reports that this fungus can produce microsclerotia.

A low level of interest has continued, primarily in South Asia, but there do not seem to be any commercial products at present. Ground maize coated with molasses was the best solid medium for conidial production evaluated by Maimala et al. (1999). Sreerama Kumar et al. (2005) focused on submerged liquid fermentation and observed that low concentrations of polyethylene glycol in a dextrose peptone medium allowed the production of mycelial pellets that were competent to produce conidia. A powder formulation, containing both mycelia and conidia, was developed in India during the 2000s, followed by two liquid formulations, but efforts to commercialize were discontinued (Sreerama Kumar, 2010). Blastospore production (mixed with abundant mycelium) is possible using Romero-Rangel et al. (2012). Their study yielded a maximum of 4×10^7 blastospores and 70 mg biomass /L, but only after 14 days' fermentation. Media designed for *Beauveria* spp. or *Metarhizium* spp., as described in Section 11.2.6.2.2 need to be evaluated. Undiluted coconut water was equivalent to two standard laboratory liquid media (potato dextrose broth, glucose yeast extract broth) in a surface-mat-on-liquid fermentation, but very inferior to Sabouraud dextrose broth, which produced 5.1×10^5 spores/cm^{-1} fungal mat surface (Mohan et al., 2016), a yield practical only for artisanal production. It would be interesting to determine the yield from coconut water plus a nitrogen source on an inert substrate.

11.2.6.4.4 Metarhizium (Nomuraea) rileyi

Metarhizium (*Nomuraea*) *rileyi* (Farl.) Samson, a pathogen of certain Lepidoptera, has undergone relatively little development and commercialization. De Faria and Wraight (2007) identified only one product, which is now no longer being marketed. There seem to now be several products. Very recently this species has been transferred to the genus *Metarhizium*. The conidia are produced on erect conidiophores much like in *Beauveria and Cordyceps*.

Both solid substrate and submerged liquid fermentation have been explored for mass production of this fungus, primarily in Latin America, South Asia and China. Rice has been identified as an excellent substrate, although best conidial yields require precooking in boiling water versus simple autoclaving (Méndez et al., 2010); the boiling presumably gelatinizes the starch in the rice, making it more available to the fungus. The liquid phase of their biphasic system used molasses and yeast extract. In India Vimala Devi et al. (2000), Kulkarni and Lingappa (2002) and Lalitha et al. (2008) identified sorghum and rice grains as the solid substrates of choice. The second group also supplemented their grain with 1% yeast extract for better conidial production. Vimala Devi et al. (2000) determined that 2% barley extract and 1% soybean extract provided cheap C and N sources for the liquid production phase, while sorghum seeds with 0.5% yeast extract served as the best solid substrate. They also noted that good aeration of the solid substrate was essential and closed plastic bags prevented conidiation. In evaluating a variety of agricultural products and byproducts, Tincilley et al. (2004) identified sugarcane spent wash liquid medium (still culture), rice, finger millet and groundnut cake as suitable solid substrates. Thakre et al. (2011) continued examination of alternative substrates; rice, sorghum, and refuse raw bananas yielded the greatest conidial numbers. A liquid medium for submerged fermentation, consisting of molasses and yeast extract was developed either for still culture, or to produce inoculum for rice gruel semisolid substrate (Ramegowda et al., 2007). In Columbia, a mass-production system was devised using plastic bags with autoclaved mijo (millet) grains, inoculated with 8-day-old fragments of agar grown with the sporulated fungus (Villamizar et al., 2004). Illumination was essential for conidiation in at least one strain of *M. rileyi* (JianWen et al., 2009), yet Bell (1975) stated that light had no effect on the growth or sporulation of an unidentified *M. rileyi*. This discrepancy may be due to strain differences.

11.2.6.4.5 Aschersonia

Aschersonia spp. are specific to Aleyrodidae (whiteflies) and Coccoidea (scale insects), and because of the growing impact of the former insect, the genus, and particularly the species *A. aleyrodis*, has received periodic attention in the past by researchers, for example, Ramakers and Samson (1984); Fransen and van Lenteren (1993). It frequently causes severe epizootics in these insects in the tropics and subtropics and was one of the first fungi used in inoculative biocontrol, when, in the early 1900s, US citrus growers introduced infected insects into their orchards. One European company briefly considered its commercialization but did not do so. An overview of the biology of *A. aleyrodis* is given by Fransen (1990) This fungus differs from the other Ascomycetes because the aerial conidia are produced within pycnidia rather than on exposed structures.

Very little work on the mass production of this fungus has been published, and most research has focused on *A. aleyrodis* rather than the other species in the genus. Ibrahim et al. (1993) observed that semisolid rather than liquid media were better for growth and sporulation, with macerated pumpkin being the best of the media they tested. They also observed that the fungus sporulated well on the surface of still liquid culture, a process developed by Czech scientists for *B. bassiana* (Bartlett and Jaronski, 1988). Zhu et al. (2008) studied the nutritional requirements of one strain of

A. aleyrodis in liquid culture (for mycelial biomass) and on agar media (for conidial production). They thereby identified a semisynthetic liquid medium of soluble starch, tryptone, Ca^{2+} and folacin, and a solid medium of lactose, tryptone, Fe^{2+}, and vitamin B_1. The use of an orthogonal matrix method allowed Zhu and coworkers to define the concentrations of each medium component. Very recently optimal solid substrate medium for *Achersonia placenta* Berk. was identified by Response Surface Method analysis to contain millet, KH_2PO_4 $MgSO_4$, albeit in an agar medium (Qiu et al., 2013).

11.2.6.4.6 Culicinomyces

Culicinomyces clavisporus Couch was investigated beginning in the 1980s as a biocontrol agent for the control of larval mosquitoes (Sweeney, 1985. Since then, however, interest in this fungus seem to have waned, probably because of the commercial success of *Bacillus thuringiensis israelensis* Berliner (Bti) and *Lysinibacillus* (*Bacillus*) *sphaericus* Meyer and Neide for mosquito control. One exception was a small resurgence regarding its potential to control biting midges (*Culicoides* spp. Latreille) reported by Unkles et al. (2004), but there is little published literature on the topic. Conidia have been produced on a wheat-bran solid substrate, or in liquid media (cornmeal extract, corn steep liquor, or standard nutrient broths) but yields were very low in comparison to efficacious field rates. The fungus has been experimentally grown in 750−1000 L fermenters in Australia and harvested by filtration or centrifugation (A. Sweeney, personal communication), but no details about the process are known to the author).

Additional experimental production using the marcescent process as described earlier for Entomophthorales was evaluated by Roberts et al. (1987) and Goettel et al. (1984). Mycelia were produced in liquid peptone-yeast extract-glucose medium, harvested by filtration treated with 10% sucrose, and air-dried to 13% moisture, then granulated. While freshly dried and granulated marcescent mycelium produced abundant conidia that could be stored at room temperature or 4°C, the product lost viability within 2 weeks. Mycelium stored at −20°C did retain viability at least for 63 days. Given the biology and ecology of *Culicinomyces* this fungus has potential as a persistent biological control agent for mosquitoes and perhaps *Culicoides* spp. but considerable technical advances in mass production are still necessary to achieve success.

11.3 Process and quality control in mass production

A quality mycoinsecticide or fungal biocontrol agent is critical for successful use. A quality mycoinsecticide demands a thorough quality assurance component to any production effort. Despite what ostensibly seems to be fine control of fermentation variables, the production process be it liquid or solid substrate, is only semicontrolled, especially when on a large commercial scale (1000 + L liquid fermenters, 10,000 kg solid substrate fermentation batches). For example, during the initial commercial production of *B. bassiana* GHA by Mycotech in the mid-1990s we observed meaningful differences in the shelf life of dry ($a_w < 0.35$) conidial powders from 16 full-scale production runs, with LT_{50}s (time to loss of 50% of the original conidial viability) at 25°C ranging from 180 to 700 days. By 1999, after a new production facility had been established, the half-lives of conidial powders ranged from 280 to 450 days (25° C.). These powders were produced under closely controlled environmental conditions and dried to 5%−7% moisture ($a_w = 0.25–0.30$). To ensure that a quality product will be produced over the long term, it behoves the mycoinsecticide enterprise to constantly monitor agent viability, physical specifications (like moisture), contamination levels, as well as virulence and shelf life. Vigilance is paramount. See Bateman (2006), Jenkins et al. (1998), Inglis et al. (2012), and Jaronski and Jackson (2012), for a discussion of specific quality control parameters and methods.

There is another problem in mass production efforts—genetic changes (degeneration) in the fungus. Butt et al. (2006) expressed the situation succinctly, "Entomogenous fungi will degenerate when continuously cultured on nutrient-rich media." Genetic changes can encompass virulence determinants, changes in colony color and gross morphology, and decline in conidial or metabolite production. Some of the fungi can give rise to morphologically different sectors in radial colonies. I have found this particularly true of *Metarhizium* spp., less so with *B. bassiana*. Butt et al. (2006) present a detailed discussion of this topic and the interested reader should refer to this work.

Changes in virulence with repeated subculture vary widely, depending on the fungus species and strain. Butt et al. (2006) have summarized all the reports up to 2006. Since then, additional studies have been performed by Hussain et al. (2010), Shah et al. (2007), Ansari and Butt (2011), Rajanikanth et al. (2011), and Safavi (2011, 2012). Under commercial conditions bioassays of 35 standard production runs of *B. bassiana* GHA, spanning 2 years, did not reveal any significant changes in virulence (LD_{50}) for the migratory grasshopper, *Melanoplus sanguinipes* (F.), nor did eight production batches for nymphal *Bemisia tabaci* (Gennadius) (Jaronski, unpublished data).

It is possible to restore, at least partially, lost attributes by in vivo passage through an insect (Shah et al., 2005). Other examples are given in Butt et al. (2006). In the course of selecting for improved heat tolerance of an *M. anisopliae* strain via continuous growth under selective conditions, the ability to sporulate was lost in two heat-tolerant clones but regained after one passage through a grasshopper (de Crecy et al., 2009); virulence was only partially restored.

Nevertheless, care must be taken to minimize the number of in vitro conidiation cycles. Typically, in an industrial situation, multiple "mother" cultures are prepared from the second or third in vitro passage from an insect and preserved by freezing with cryoprotectants to $-80^{\circ}C$ or lyophilized. From this mother culture, enough subcultures are prepared for 6 months or a year of production cycles and frozen until used. When these are exhausted, another mother culture is thawed and the cycle is repeated.

There is another aspect to potential genetic changes—the number of mitotic divisions and propagules generated during the typical production cycle. Consider that in one commercial production process an initial inoculum of 1×10^9 conidia results in 4.5×10^{16} conidia at the end of a biphasic production cycle, a 4.5×10^7-fold multiplication. Yet, no changes in the fungal RFLP pattern were noted, indicating considerable genetic stability (Jaronski, unpublished data).

11.4 Current knowledge about the effect of cultural conditions on propagule attributes

Fungal spores (blastospores and conidia) have a proscribed environmental range that affects their infectivity (efficacy). Spores require high humidity for germination, although this requirement can be obviated by the microhabitat of the insect cuticle or phylloplane boundary layer or be modified with formulations. In addition, spores can be subjected to desiccation in the target habitat following application in an aqueous spray. Conidial germination, as well as vegetative growth within the insect, is limited in both speed and extent by high ($>32^{\circ}C$) and low ($<15^{\circ}C$) temperatures. The speed of conidial germination even at optimal temperatures can be critical with frequently molting insects such as aphids. UV-A (315−406 nm) and UV-B (280−315 nm) are lethal to conidia, greatly shortening their persistence in the foliar habitat, and limiting efficacy when the target insects are more likely to acquire spores from treated habitat rather than direct spray. See Jaronski (2010) for a full discussion of ecological considerations in the inundative use of entomopathogenic fungi.

The different fungus species and isolates within each species vary in their tolerances to these environmental factors (Devi et al., 2005), as well as in virulence and mass production potential. Typical development programs thus seek to identify the "best" fungus for a particular use and target, and formulations are devised to accommodate fungal deficiencies to a great or lesser degree of success. In recent years, there have been attempts to improve efficacy through genetic modifications. A developmental program can therefore expend considerable effort in screening candidate fungi, emphasizing virulence, spore production, and shelf life, and optimizing fermentation variables to maximize spore production.

There may be another approach, at least to improve an already acceptable fungal agent—manipulation of fermentation conditions to improve environmental stress tolerance. Magan (2001) posed four key questions on this subject, questions that are still relevant: "(i) can one manipulate the physiology of nonxerophilic/tolerant fungi to accumulate useful endogenous reserves into inocula for improved environmental stress tolerance? (ii) would this result in improved germination/growth under environmental stress? (iii) can this improve the establishment of inocula and conserved biocontrol potential in the field? and (iv) does ecophysiological manipulation have a role in improving the production and quality of inocula?" Magan (2001) pointed out that there are xerotolerant/xerophilic fungi able to tolerate a range of water availability that inhibit the entomopathogenic species, which are inhibited by water activities below 0.95−0.93 a_w. The ability to tolerate water stress conditions in these fungi is associated with compatible solutes within the spores. The potential for such manipulations was identified by Matewele et al. (1994), who observed that mutants of *M. anisopliae* and *C. farinosa*, which were able to germinate and grow at lower water activities than parental strains and which were subsequently grown on low water activity medium ($a_w = 0.969$), were more virulent against green leafhopper than the parental strains. Also, *B. bassiana* conidia, having an increased glycerol and erythritol content, germinated in low a_w (0.935) media but conidia having low glycerol + erythritol failed to germinate below 0.951 a_w (Hallsworth and Magan, 1995). In addition, Magan (2001) noted that *M. anisopliae* conidia from an insect had significantly greater polyol content and different sugar/polyol ratios than conidia produced on an agar medium. These data strongly suggest that endogenous solutes can be manipulated. Can manipulation affect stress tolerance and improve efficacy? While there is not an abundance of data on this subject, what does exist poses intriguing possibilities.

11.4.1 Age of conidia

Hall et al. (1994) observed that young conidia produced by all the isolates of *M. anisopliae* and *Lecanicillium* tested germinated faster than older ones, whereas *B. bassiana* conidia germinated at the same rate, regardless of culture age.

The impact of culture age on conidial germination appeared to be species and strain-dependent. More recently, Smith and Edgington (2011) showed that the capacity of conidia to withstand water stress developed by low water activity was related to the age of fungal cultures, implying that a prolonged production cycle (conidial ageing) may also improve the fitness of conidia.

"Old" spores (collected after 14 days of fungal growth) of *B. bassiana* and *M. anisopliae* produced on agars composed of whey permeate or millet were more thermotolerant than conidia from quarter-strength Sabouraud dextrose yeast agar, although the differences were inconsistent among the two isolates of each species Kim et al. (2010a). A mixed message was described by Rajanikanth et al. (2011), whereby conidia of six strains of *B. bassiana* from 14-day-old cultures had greater virulence for larval *Spodoptera litura* F. than conidia from 7, 21, 28-day-old cultures.

A clue to what may be going on with age-related differences in conidia is offered by Kim et al. (2010a), who observed that two isolates each of *B. bassiana* and *M. anisopliae* had conidia of two types, having a different degree in hydrophobicity, and termed young (7-day-old) versus old (mature, 14-day-old) conidia. Thermotolerance seems to have been directly associated with hydrophobicity (age) of the conidia, although there were differences among the isolates and media used to generate the conidia. Quarter-strength Sabouraud dextrose yeast agar had the most pronounced difference between the two spore ages.

11.4.2 Conidia produced under certain nutrient conditions or under osmotic stress

Conidia of *M. anisopliae* and *I. farinosa*, produced on agar media adjusted to an aw of 0.96, were significantly more infective for *Galleria mellonella* (L.) larvae at 86% and 78% (Hallsworth and Magan, 1994b). The series of studies by Hallsworth and Magan (1994a, 1994b, 1994c, 1995, 1996) were significant in that they lay the foundation for the manipulation of fermentation media to change the solute content of conidia and thereby improve desiccation tolerance. There are inter- and intra-species differences in the response to particular growth conditions Hallsworth and Magan (1999). Overall, however, lowering a_w below 0.98 reduces mycelial growth and conidial yield, so there is a fine line between obtaining xerotolerance and not seriously affecting spore yield. Also, it should be kept in mind that these studies were conducted with agar media; it may be difficult to control a_w within fine tolerances on a solid substrate, especially when in large quantities under commercial conditions.

Lane et al. (1991) examined the effect of C:N ratios on blastospore production by *B. bassiana*. They observed, first, that inorganic nitrogen-limited blastospores had a longer shelf-life than blastospores from carbon-limited media, which was accompanied by differences in morphology and endogenous reserves. Second, while there were no differences in the LC50s of carbon- and nitrogen-limited blastospores for a leafhopper, the virulence (LT50) of nitrogen-limited blastospores was greater than their carbon-limited counterparts and the former adhered more firmly to insect cuticles than the latter.

Virulence of *M. anisopliae* conidia seems to be slightly affected by the C:N ratio of the (agar) medium, with the most virulent conidia having an endogenous C:N ratio $<$5.2:1, per the claims of Shah et al. (2005). The conidial C:N ratios were affected by the agar medium used, with a glucose: peptone medium having a C:N ratio of $>$35:1, or an osmotically stressful medium, via KCl addition, causing the greatest endogenous C:N ratio. However, virulence for *Tenebrio molitor* L. was unaffected from a biological perspective, varying from 3.5 to 4.1 days for one strain and 3.5 to 3.9 days for a second strain. A problem with interpreting these data is that virulence, as time to 50% mortality, is an expression of mycelial development rather than conidial fitness per se.

In a related study, but with *B. bassiana*, Safavi et al. (2007) did not observe the effect of endogenous C:N ratio as did Shah et al. (2005) with *M. anisopliae*. While endogenous C:N ratio was affected by the different media, Safavi and his associates could not see a clear relationship among C:N ratio, germination rate, conidial PR1 activity and virulence in their *B. bassiana* isolates. Osmotic stress caused the lowest conidial production, however.

Rangel et al. (2004) noted that conidia of two isolates of *M. anisopliae* obtained from insect cadavers were more sensitive to UV-B irradiation than those produced on a rich artificial medium, and they also germinated more slowly. The artificial medium also had an effect, with conidia from rice substrate or two agar media having more UV-B tolerance than conidia on potato dextrose yeast agar. Rangel et al. (2004) subsequently observed that UV-B tolerance of *M. robertsii* conidia was increased at least twofold when the fungus was grown on agar medium containing nonpreferred carbon sources, such as fructose, galactose, lactose, versus preferred carbon source, such as dextrose. With lactose, endogenous trehalose and mannitol accumulated to a significantly greater level. Conidial yields were, however, reduced, which could be a considerable disadvantage.

In follow-up work, Rangel et al. (2008a, 2008b) examined the effect of growing *M. robertsii* under different stress conditions. Conidia produced on a minimal agar medium, and minimal medium supplemented with lactose had the

fastest germination and greatest bioassay virulence for *T. molitor* than conidia from a rich medium (potato dextrose agar with yeast extract). These researchers also observed that conidia produced under conditions of carbon or nitrogen starvation possessed significantly greater heat and UV-B tolerance than conidia produced on nutrient-rich PDAY. The greater tolerances were associated with the greatest accumulation of trehalose and mannitol within the conidia. Similar results were seen with an osmotically stressful agar medium (0.8 M KCl or NaCl), a_w unknown. Conidial yield, however, could be severely affected by stressful conditions during mycelial growth.

Thermotolerance (conidial viability after exposure to 48°C. for 30 minutes) seems also to have been significantly increased when glucose (4% optimal) or starch (1%) were the carbon sources in agar media (Ying and Feng, 2006). When $\leq$ 50-μg/mL Mn^{+2} was added, thermotolerance was extremely good and was greatly increased with sucrose and Fe^{+3} (Ying and Feng, 2006). The pH had some effect; thermotolerance was greatest when the fungus was grown at pH 5−6; osmotic stress, in the form of KCl, had an adverse effect. Amendments, such as Mn^{+2}, or carbon supplementation, with a grain-based solid substrate, are feasible, but again, whether this phenomenon extends to other strains or species remains to be elucidated.

An insight into the mechanisms of thermotolerance may be gotten from Leng et al. (2011). Using RNAi, they demonstrated that trehalose levels in *M. acridum* conidia were closely associated with tolerance to heat stress. There also seems to be a relationship between thermotolerance and formic-acid-extractable proteins in aerial conidia of *B. bassiana* and *C. fumosorosea* (Ying and Feng, 2004). The formic-acid-extractable proteins are affected by the nature of the carbon source, glucose and sucrose causing significantly better thermotolerance than starch (in an agar medium).

Investigations about a_w effects can also be extended to solid substrate media. The a_w of rice substrate (modified by the use of different normalities and amounts of HCl) affected the polyol content of *B. bassiana* conidia and the relative proportions of mannitol, arabitol, glycerol and erythritol (Tarocco et al., 2005). The last two are the most biologically important − high intraconidial concentrations of glycerol and erythritol were associated with the ability of conidia to germinate at much lower a_w than otherwise (Hallsworth and Magan, 1994b, 1995). The optimal a_w for glycerol/erythritol accumulation was 0.980. Conidial production, however, was severely reduced, by 95%−99.8% (optimal a_w for conidial production was 0.999). A "compromise" a_w, yielding acceptable polyol levels still resulted in a 72% reduction in yield.

Kim et al. (2011) discovered that millet grain as a solid substrate produced *B. bassiana* and *M. anisopliae* conidia that were more thermotolerant (to 43°C−47°C) than conidia from agar-based media. Their data indicate that certain substances in the millet contributed to the observed thermotolerance. The use of millet supplemented with plant-derived oils, especially corn oil, further induced greater heat tolerance of *B. bassiana* conidia (45°C for 90 minutes) (Kim et al., 2010c). In a subsequent paper, Kim et al. (2010b) demonstrated that, for *I. fumosorosea*, ground corn solid substrate produced conidia with the most heat tolerance, compared to rice, soybean of red kidney bean substrates, and corn oil supplementation of the corn meal increased that heat tolerance (Kim et al., 2010a). They hypothesized that unsaturated fatty acids, such as linoleic acid and oleic acid, in the corn oil possibly explained the improved thermotolerance.

The addition of 1−4 mM salicylic acid, a plant cell-signaling metabolite, to an agar medium yielded conidia of one *M. robertsii* isolate with a doubling in heat tolerance, but not UV-B tolerance (Rangel et al., 2012). It should be noted that the amendment did reduce conidial yield somewhat and a defined, agar-based was used in the study. Whether the same phenomenon would result in either solid substrate or submerged liquid fermentation, or with other *Metarhizium* spp. strains, species and *B. bassiana* remains to be determined. Many of the fermentation manipulations caused a significant decrease in spore production, to the extent that the manipulations were self-defeating. Nevertheless, further research may find a satisfactory compromise between stress tolerance and spore production.

Addition of riboflavin to *Metarhizium* spp. grown on agar medium evidently increased the UV-A or UV-B tolerance of the resulting conidia (Pereira-Junior et al., 2018; Huarte-Bonnet et al., 2020). However, several attempts to replicate this study, using *B. bassiana* and *M. anisopliae* grown on rice or flaked barley solid substrate, failed to demonstrate any change in the UV sensitivity of the resulting conidia (Jaronski, unpublished data).

11.4.3 Conidia produced after photoirradiation during vegetative growth

Intriguing information exists about the effect of light on *Metarhizium* spp. during vegetative growth, effects manifested in changes in environmental tolerances of the resulting conidia. Conidia produced from mycelium of *M. robertsii*, irradiated with UV-A while growing on PDAY, had somewhat elevated virulence levels similar to that of conidia produced on nutritionally minimal medium, but their germination rate was not increased, nor were UV-B resistance and thermotolerance improved (Rangel et al., 2008a, 2008b; 2011). Agar cultures of *M. robertsii* incubated under white light at various intensities developed conidia with increased tolerance to osmotic and oxidative stress (Dias et al., 2021, 2022). The

color of the light seems to have a differential effect on conidial germination under stressful osmotic conditions, and also greater tolerance to UV-B (Dias et al., 2020). In addition, *M. robertsii* conidia produced by agar cultures grown under blue or white light germinated faster and evidently demonstrated a greater virulence for Tenebrio larvae in bioassay (Oliveira et al., 2018). Illumination of vegetative mycelium needs further investigation to determine the applicability of the observations to other strains and other species., Here, illumination of the fungus grown on broken rice in small quantities within polypropylene bags resulted in increased conidia production compared to constant darkness, although the best yields were a small fraction of what others have reported (Bich *et al*., 2018). All of the studies quoted here except the last involved fungus cultures on agar media, not solid substrate mimicking production, so the practicality of many of these findings, while informative about the basic biology of these fungi, is unclear. Most fungus mycelium growing in a large mass of solid substrate whether in a plastic bag or in a tray within an incubator cannot be easily illuminated because light cannot penetrate more than a few mm into a mass of substrate.

11.5 The challenge in mass production of entomopathogenic fungi

The fundamental consideration in the potential commercialization of any of these fungi is whether an efficacious product can be developed and produced cheaply enough to compete with existing controls, especially chemicals. To return an acceptable profit on the research and development investment, low-cost mass production of an entomogenous fungus is only one of several technical constraints. Yet, efficient, economic mass production is basic to commercialization.

There is relatively little published data about the economics and efficiencies of commercial production. Such data are obviously proprietary. There are academic studies comparing liquid and solid substrate production but these rarely if ever take into account the many economic components of industrial production. A comparison of the two processes for producing *A. muscarium* was presented by Ravensberger (2011). For the manufacturer, liquid submerged fermentation yielded 2.1×10^{13} spores/L, within 7 days, in 1000 L fermenters, while solid substrate fermentation in specialized equipment of 100 kg substrate capacity yielded 8.1×10^{12} conidia/kg. Ravensberger (2011) estimated that new solid substrate equipment would cost €750,000 in 2007 versus €300,000 for a 1000 L liquid fermenter that had 4 times the production capacity. Inflation in the intervening 14 years increases these sums. Ignoring the quality of spores, viability losses to desiccation, production of unwanted metabolites in submerged fermentation, and subsequent shelf life, that submerged liquid fermentation may be the more practical. However, these aspects can render the economics of production unprofitable.

The scale of production is one key to the level of technology used. Bartlett and Jaronski (1988) examined the capacities of the different technologies at the time and concluded that high efficiency, mechanized, biphasic solid substrate, such as now practiced by at least one company in the US, was the only commercially feasible system for very large mycoinsecticide production needs. For example, consider a potential US market, European corn borer, *Ostrinia nubilaris* (Hübner), in maize, ignoring for the moment the dominance of Bt-maize. In 2020, 34 million ha of maize in the US was subject to attack by European corn borer, *Ostrinia nubilalis* (Hubner), and corn earworm, *Helicoverpa zea* (Boddie) (US Department of Agriculture, National Agricultural Statistics Service, 2021). Assuming 5% of the corn market is open to mycoinsecticide use, that is 1.7 million ha. Much of the published literature on efficacy indicates a rate of $1-2.5 \times 10^{13}$ conidia ha^{-1} is needed for fungal products (De Faria and Wraight, 2007). If the use rate of a mycoinsecticide is a more optimistic 5×10^{12} conidia/ha, then a company would need to produce 8.5×10^{18} conidia for one growing season. With the public Mycotech/Laverlam technology yields of 2.6×10^{13} conidia/kg (Bradley et al., 1992), that need would require 327,000 kg of substrate. If a production run involved 10,000 kg using a highly mechanized system, then 33 fermentation runs would suffice in a year. Lower yields, such as reported in the literature, or higher rates of fungus for satisfactory efficacy, would require much larger amounts (Table 11.4).

In contrast, for smaller-scale production, serving local needs, solid substrate fermentation, for example, in plastic bags, as is practiced in Cuba, Brazil and other Latin American countries, may be practical. For 1000 ha, based on an application rate of 5×10^{12}/ha, about 5×10^{15} spores would be needed. Production using 3300 kg substrate (with a yield of 1.5×10^{12}/kg) would meet that need, ignoring loss to contamination, less than 100% recovery, and less than 100% conidial viability (a common occurrence, esp. with *Metarhizium* spp.). If the highest yield obtained on a lab-scale in plastic bags, 2×10^{13}/kg rice (Dorta et al., 1996), was operationally possible; then, the substrate need would decrease to 250 kg, which is much more feasible for a small operation. Another example is given by Posada-Flores (2008). Nevertheless, use of plastic bags, a common method, can be a limiting factor in meeting large needs.

For inoculative release of fungi in a biocontrol campaign, for example, with Entomophthorales, such considerations are avoided; only small numbers of spores are needed because the fungus will reproduce and proliferate, ideally causing an epizootic. In inundative applications, the fundamental challenge is to reduce fungus use rates. While the typical

TABLE 11.4 Estimates of production capacity needed to supply enough fungus propagules to treat 1.3×10^6 hectares of maize in the US, 5% of the total potential maize market based on documented production yields.

Fermentation system	Yield per unit	Fungus (source)	Production need
High efficiency biphasic solid substrate fermentation	2.5×10^{13} conidia/kg substrate	*B. bassiana* GHA (Bradley et al., 1992)	2.6×10^5 kg
Low efficiency (bag) solid substrate fermentation	1.5×10^{12} conidia/kg	*M. acridum* IMI330189 (Jenkins et al., 1998)	4.3×10^6 kg
Submerged fermentation	$1-2.8 \times 10^{12}$ blastospores/L	*C. fumosorosea B. bassiana* (Mascarin et al., 2018)	$2.3-6.5 \times 10^6$ L
Liquid surface culture	1×10^{14} conidia m^{-2} of surface	*B. bassiana* (Bartlett and Jaronski 1988)	6.5×10^4 m^2

Note: The total spore need is 6.5×10^{18} spores, based on a use rate of 5×10^{12} spores ha^{-1}.

application rate is on the order of $1-2 \times 10^{13}$ spores/ha, lower application rates have been obtained by the selection of a more efficacious (yet still productive) strain. The official use rate for *M. acridum* against locusts is 5×10^{12}/ha, already a lower rate than the generally accepted level for a mycoinsecticide, and good efficacy has been obtained with rates as low as 1.25×10^{12}/ha. One-fourth of the fungus per acre has a major impact on production needs. In the past few years' efforts have been made toward creating fungal strains genetically modified for greater virulence (reviewed by Lovett and St Leger, 2018). Some of these transformations have greatly increased the efficacy of the fungus, for example, the incorporation of scorpion neurotoxin increased toxicity for hornworms 22-fold (Wang and St Leger, 2007). If regulatory agencies will allow the use of such transformed fungi, application rates could be considerably lowered. So far, no regulatory agencies have.

Use rates per unit area have also been lowered by concentrating the spores into a narrow, targeted zone by modification of application equipment and methods to deliver more spores exactly where needed (Jaronski, 2010). If applied in a broadcast spray, 5×10^{12} conidia/ha would result in a level of 5 x 10^4 conidia cm^{-2} of surface area. With a 12.5 cm banded application of each row of plants, the conidial levels become $2.4 \times 10^5/cm^{-1}$, a fivefold increase at the same rate per hectare, confined to the actual habitat of the target insect, or a potential fivefold reduction in the rate of fungus per hectare if the 5×10^4 conidia/cm^{-1} of surface area was efficacious. For example, Wraight and Ramos (2002) were able to increase the conidial deposition on leaf undersides 6- to 30-fold by modifying the arrangement of spray nozzles. Similarly, spores can be placed in the path of insects, such as on fiber bands wrapped around tree trunks to control the Asian Longhorned Beetle, *Anoplophora glabripennis* Motschulsky (Shanley et al., 2009), or in compact ultra-low volume spray bands of *M. acridum* applied in front of migrating locusts, affording considerable economies. Another approach is to bring the target insect to the fungus through the use of pheromones, and attractants (e.g., Akutse et al., 2020; Mfuti et al., 2021). There is considerable potential for fungi to be significant tools in managing insect pest populations, other than broadcast sprays needed large quantities of spores.

A concept that is often lost in the development of a mycoinsecticide is that any microbial, regardless of its nature, is only one tool in an IPM system. With that in mind, the use of a microbial, in the present case a fungus, complements other tools (culture, mechanical, plant resistance, macrobiological, chemical). The mycoinsecticide does not have to achieve control levels of a chemical pesticide. The key is using a microbial, with other practices, to lower the pest insect population below the economic threshold for that crop.

With the increased interest in biological pest control approaches, efficient, economic production of entomopathogenic fungi has generated a lot of scientific effort, achieving considerable advances in the past 10 years. I wonder what new discoveries to enhance greater adoption of entomopathogenic fungi, along with other microbials, lie in wait.

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Chapter 12

Commercial production of entomopathogenic bacteria

Terry L. Couch[1], Trevor A. Jackson[2] and Juan Luis Jurat-Fuentes[3]

[1]*Becker Microbial Products Inc., Parkland, FL, United States,* [2]*AgResearch, Lincoln Research Centre, Private Bag 4749, Canterbury Region, Christchurch, New Zealand,* [3]*Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN, United States*

12.1 Introduction

Bacteria are generally defined as unicellular and ubiquitous microbes possessing a single chromosome not surrounded by a nuclear cell membrane and having 70S ribosomes (Jurat-Fuentes and Jackson, 2012). These organisms proliferate through binary fission, a process resulting in daughter cells that are essentially identical copies of the mother cell which makes them amenable to mass production through growth on suitable media. Among the bacteria, some species and strains have evolved as entomopathogens causing infection and death to the host. This property has been recognized as useful in the control of agricultural pests and disease vectors and has led to the development of bacteria as biopesticides (Siegel, 2000). The focus of this chapter will be on large-scale production methods for bacterial species lethal to insects that are sold as biopesticides.

The development of an entomopathogenic bacterium as a biopesticide will firstly depend on whether there is a commercial market, as considerable financial resources are required to scale up, produce and formulate an entomopathogenic bacterium on a commercial scale. The challenge is then to produce a biopesticide product which can be produced in bulk and distributed to meet the needs of the market. While entomopathogenic bacteria can be widely found in nature (Abdelgaffar et al., 2022) only a small number of species and strains have been considered for commercialization and even fewer have led to successful products. The greatest commercial success has been achieved with bacteria from the Gram-positive genera *Bacillus* and *Lysinibacillus*. These organisms can be grown in large scale fermenters producing spores and toxins which can be dried for a stable end product (Fig. 12.1). Gram-negative bacteria can also be produced in large volumes by fermentation but do not form spores and require formulation to provide stability.

The first commercial biopesticide (Sporeine) was developed in France in the 1930s and was based on the Gram-positive *Bacillus thuringiensis* Berliner (Beegle and Yamamoto, 1992). Moreover, the first bacterial entomopathogen used in a major insect control program was *Paenibacillus popilliae* (Dutky) (previously *Bacillus popilliae*) (Klein and Jackson, 1992), the causative agent for milky disease in the Japanese beetle (*Popillia japonica* Newman). However, *P. popilliae* is highly fastidious and, despite successful use, problems related to the mass production of viable *P. popilliae* spores (Stahly and Klein, 1992) reduced commercial interest and current use is limited to the control of grubs, especially in organic agriculture (Jackson, 2017). Currently, the most commercially relevant Gram-positive entomopathogenic bacteria are *B. thuringiensis* and *Lysinibacillus* (formerly *Bacillus) sphaericus* (Meyer and Neide) which will be the focus of this chapter.

Gram-negative bacteria have achieved less success as commercial products given their shorter stability. Among the Gram-negative entomopathogens, *Serratia entomophila* Grimont et al. has been developed as a live biological control product for the New Zealand grass grub (*Costelytra giveni* Coca-Abia and Romero-Samper formerly *C. zealandica*) (Jackson, 2007). Another Gram-negative bacterium, *Chromobacterium subtsugae* Martin et al. has been developed as a product containing live bacteria and fermentation residues to control a range of insect pests (Martin et al., 2007). Many other nonspore-formers such as *Yersinia entomophaga* Hurst et al. and *Pseudomonas entomophila* Mulet et al. have shown excellent insecticidal activity, but have yet to be developed as biopesticide products (Hurst et al., 2011; Vodovar et al., 2006).

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FIGURE 12.1 Large scale fermenter vessels at E-Nema GmbH, Schwentinental, Germany. *Photo Dr Arne Peters.*

The challenge of meeting market fit has meant that few entomopathogenic bacteria have reached a commercial scale of production despite the advantages of deep tank fermentation and little published literature is available, as the majority of data dealing with actual scale-up of commercial products based on entomopathogenic bacteria are proprietary material. Information to provide practical advice on production and formulation in this chapter will mostly rely on the topic reviews from Beegle et al. (1991), Lisansky et al. (1993), Ravensberg (2011), and Jackson (2017) and the practical experience of the primary author during his career working on large scale production of biopesticides.

12.2 Biology of commercial entomopathogens

Both *B. thuringiensis* and *L. sphaericus* are ubiquitous soil microbes, and isolates have been obtained from multiple environments worldwide (Guerineau et al., 1991; Bernhard et al., 1997). Main phases of vegetative (exponential) growth and sporulation (stationary) can be easily differentiated in the life cycle of both species, which are also observed during commercial production. Vegetative cells (Fig. 12.2A) are bacilliform (rod shaped) and in some cases highly motile due to the existence of flagella. These cells divide by fission and grow exponentially until nutrients are depleted or adverse environmental conditions occur. At this time, specific genes are activated that drive sporulation and synthesis and formation of crystalline protein parasporal inclusions (Fig. 12.2B). Once sporulation is completed, sporangia lyse and the endospore and crystal are released into the medium. These parasporal crystals contain proteins responsible for insecticidal activity, which are classified and named according to their amino acid sequence identity and structural similarity (Crickmore et al., 2020).

Despite their bacilliform shape during the vegetative phase, *B. thuringiensis* and *L. sphaericus* can be easily distinguished after sporulation by the position of their endospore and the sporangium shape. Thus, while *B. thuringiensis* produces an ovoidal spore that does not deform the shape of the cell (sporangium), *L. sphaericus* produces a spherical endospore that locates in a terminal position, resulting in a swollen sporangium. The crystals may represent up to 20%−30% of the dry cell weight and are composed of diverse insecticidal proteins that are responsible for entomotoxicity. Consequently, improvement of these insecticidal proteins and production of recombinant strains with chimeric insecticidal proteins have been of interest to develop biopesticides with increased efficacy (Baum et al., 1999). Different crystal morphologies, depending on protein composition, have been described, with bipyramidal and spherical being the most common shapes (Bernhard et al., 1997).

Diverse methods have been used to classify *B. thuringiensis* and *L. sphaericus* isolates, with flagellar H-serotyping being the most widely reported in the literature. For *B. thuringiensis* (*Bt*) 85 serotypes have been described (Jurat-Fuentes and Jackson, 2012), although most commercial biopesticides are based on serovars (or subsp.) *kurstaki* (*Btk*), *aizawai* (*Bta*), *israelensis* (*Bti*), and *morrisoni bv. tenebrionis* (*Btt*). Each of these serovars includes isolates expressing crystal (Cry) parasporal proteins with entomotoxicity against specific insect taxonomic orders, including Lepidoptera, Coleoptera, and Diptera. Cytolytic (Cyt) toxins are also important for entomotoxicity of *Bti* as they synergize activity (Wu and Chang, 1985; Crickmore et al., 1995) and delay resistance (Wirth et al., 2005) to Cry proteins in mosquito larvae. Additional noncrystal toxins and other virulence factors that enhance entomotoxicity may also be produced by diverse isolates. On the other hand, the potential secretion of thermostable insecticidal toxins called beta exotoxins by some strains during the vegetative stage is of concern during commercial production of *B. thuringiensis* biopesticides.

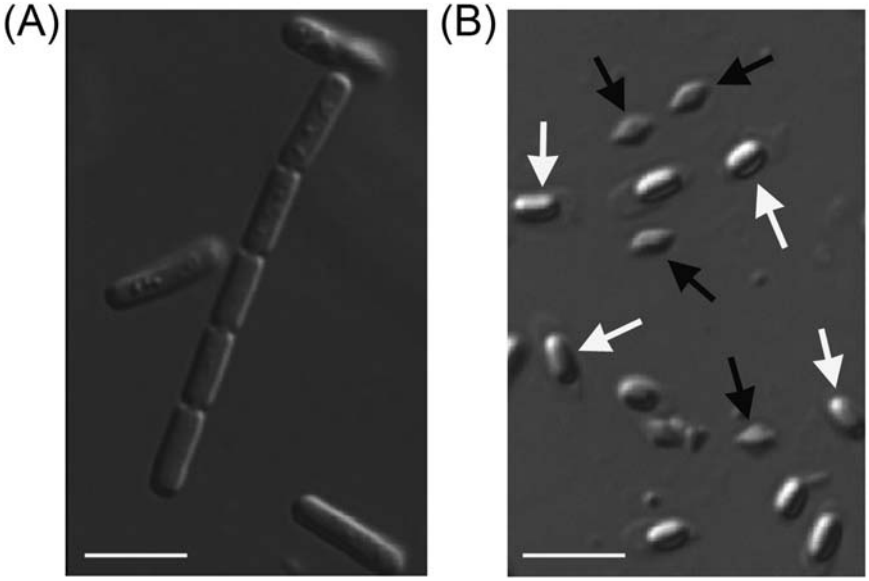

FIGURE 12.2 Vegetative (A) and sporulated (B) stages in a *Bacillus thuringiensis* subsp. *Kurstaki* strain HD-73 culture documented using Differential Interference Contrast (DIC) microscopy. White bars at the bottom of the figures represent 5 μm for reference. Vegetative cells observed during early stages (A) appear dark and bacilliform, mostly forming long chains. When nutrients are depleted cells undergo sporulation and endospores and bipyramidal parasporal crystals containing the Cry1Ac insecticidal protein may be observed inside the sporangia. Later in the sporulation stage (B) sporangia disrupt and both endospores (white arrows) and crystals (black arrows) are released and can be observed in the medium. *Courtesy of Advanced Microscopy and Imaging Center, University of Tennessee.*

These exotoxins inhibit protein production and were deemed unsafe for public use, resulting in a ban by the World Health Organization for their use in biopesticides (World Health Organization, 1999). Chromatographic purification and/or bioassays are commonly used to detect the production of these exotoxins by *B. thuringiensis* strains.

In the case of *L. sphaericus*, insecticidal activity is limited to dipteran larvae, specifically mosquitoes. Susceptibility to *L. sphaericus* amongst mosquito genera varies, with *Culex* being the most susceptible, followed by *Anopheles*, *Mansonia*, and *Aedes* (Yap, 1990). Within the 49 flagellar *L. sphaericus* serotypes initially described (de Barjac et al., 1980), only nine of them contained mosquitocidal strains. Nowadays most commercial *L. sphaericus* biopesticides against mosquito larvae are based on strains 2362 and C3−41 (Park et al., 2010), although continuous prospecting may help identify new isolates with higher entomotoxicity (Park et al., 2007). Mosquitocidal activity in *L. sphaericus* strains is mostly determined by the production of crystal binary (Bin) and soluble mosquitocidal proteins including mosquitocidal toxin (Mtx1) and pesticidal proteins with homology to the Etx_Mtx2 family (Mpp proteins).

The ecology of bacterial entomopathogens has been discussed by Jackson et al. (2018). Gram-positive *B. thuringiensis* and *L. sphaericus* entomopathogenic strains must be ingested for toxin release and bacterial colonization of the host. The production of insecticidal crystalline protein inclusions poses an energetic burden on the cells but limits their ability to grow freely in the environment in competition with other bacteria. Pathogenicity provides toxin-bearing strains with a comparative advantage in the presence of insects and the spore assists long term persistence, however, horizontal transmission is limited and natural epizootics rare. These characteristics mean that the Gram-positive agents must be applied regularly for pest control.

As with the Gram-positive agents, Gram-negative bacteria must be ingested by the target insect to have a pathogenic effect. Bacteria of several genera (*Serratia, Yersinia*, and *Photorhabdus*) produce Tc (toxin complex) toxins in the insect gut, inducing pathogenesis (Jurat-Fuentes and Jackson, 2012). Impact relies on the application of a culture of the live bacteria, which poses a challenge for the formulation and distribution of the live organisms. In contrast to the bacilli based biopesticides, the nonspore-forming *S. entomophila* is applied as an inoculative agent that persists in the pest's environment (Jackson et al., 2018).

12.3 Pathogenesis and pest control impact

Bacterial biopesticides target the larval stage of susceptible pests. Generally, susceptibility to these biopesticides decreases with larval growth (Wraight et al., 1987; Huang et al., 1999), although larvae surviving exposure often present fitness costs in later stages, such as smaller pupae that fail to develop or lower fecundity as adults (Nyouki et al., 1996; Flores et al., 2004). Moreau and Bauce (2003) showed that larvae that recover from treatment are more susceptible to sequent exposures.

The mode of action of *B. thuringiensis* insecticidal proteins (Vachon et al., 2012) identifies some common features among diverse strains and insect hosts. The generalized pathogenic process for *B. thuringiensis* biopesticides is initiated

with ingestion of the product, which contains spores and crystal insecticidal proteins as active ingredients. Typically, larval paralysis and feeding interruption are quickly observed after ingestion. The protein crystals are solubilized and processed in the midgut fluids of the larvae to activated insecticidal toxins, which interact with specific receptors on the gut cells. This interaction results in the formation of a pore on the gut cell membrane, with osmotic imbalance leading to cell death. As enterocytes die, the integrity of the gut epithelium is compromised, allowing mixing between the main body cavity (hemocoel) and the gut fluids, resulting in a lower pH that promotes spore germination to vegetative cells. The possibility that interactions between toxins and receptors also promote spore germination has also been suggested (Du and Nickerson, 1996). If damage to the gut epithelium is extensive enough to overcome any healing mechanism, vegetative cells are able to invade the hemocoel and proliferate to cause septicemia (Raymond et al., 2008). Once nutrient resources in the host cadaver are utilized, vegetative cells undergo sporulation.

While the mode of action of their insecticidal proteins differs, the main features observed for *B. thuringiensis* pathogenesis are common to mosquitocidal biopesticides based on *L. sphaericus* (Berry, 2012). Inhibition of larval feeding after ingestion of *L. sphaericus* is quickly observed, and death occurs within 2 days (Singer, 1981). The *L. sphaericus* crystals are solubilized and processed to activated toxins, which interact with specific receptors on the cells located in the gastric cecum and posterior midgut (Charles et al., 1996; Pigott and Ellar, 2007). In case of the binary (Bin) toxins renamed as Tpp pesticidal proteins in the new nomenclature (Crickmore et al., 2020), one of the toxins (BinB, Tpp2 in new nomenclature) binds to the cell surface and acts as binding domain for the other toxin (BinA, now Tpp1), which is then internalized to kill the gut cell in a process involving autophagy (Opota et al., 2011). Upon gut epithelium disruption, toxins can affect cells in the neural and skeletal tissues, explaining gut paralysis observed between 24 and 48 h (Davidson et al., 1975). After host death, *L. sphaericus* spores germinate and vegetative cells proliferate until nutrient depletion, when sporulation occurs. This amplification and release of spore numbers, together with the lower spore settling (Yousten et al., 1992), contribute to the longer environmental persistence of *L. sphaericus* compared to *Bti*. As observed for *B. thuringiensis*, sublethal exposure results in long-term effects on the development of the population (Lacey et al., 1987).

While biopesticides based on *L. sphaericus* target mosquito larvae, products based on *B. thuringiensis* target pests of Lepidoptera (butterflies and moths), Coleoptera (beetles), or Diptera (mosquitoes and blackflies), depending on the specific isolate used and the toxins it produces (Palma et al., 2014). Thus, while *Btk* produces Cry1A and Cry2 proteins, and *Bta* produces Cry1A, Cry1C, and Cry1D proteins, both serovars are active against lepidopteran insects. In contrast, *Bti* strains producing Cry4, Cry11, and Cyt toxins are active against dipteran pests (mosquitoes and blackflies), and *Btt* producing Cry3A toxin is active against coleopteran insects.

The biopesticides based on Gram-negative bacteria also target the larval stages of insects and some show extreme selectivity (Jurat-Fuentes and Jackson, 2012). For example, despite producing Tc toxins that are found among several genera of the Enterobacteriaceae, *Serratia entomophila* has only shown pathogenicity to a single species, the New Zealand grass grub (*Costelytra giveni*). After ingestion of the bacteria, the infected insect ceases feeding and clears the gut leading to a long period of chronic infection before death (Jackson et al., 2001). Further study showed that infection prevented secretion of midgut digestive enzymes through degradation of the gut cells (Gatehouse et al., 2008; Marshall et al., 2012). Another Gram-negative bacterium, *Y. entomophaga*, has a wider host range and more dramatic effect. Ingestion is followed by vomiting and gut clearance, which is rapidly followed by insect death (Hurst et al., 2014). The host range of *Y. entomophaga* is wider than for *S. entomophila*, with pathogenicity to pests from a range of insect orders. Commercialized biopesticides based on fermentation products and nonliving Gram-negative bacteria such as *Chromobacterium subtsugae* and *Burkholderia rinojensis* Cordova-Kreylos et al. are effective against a range of insect pests (Marrone, 2019).

Most commercialized bacterial insecticidal products are used in environmentally sensitive areas for crop protection and vector control. While bacterial entomopathogens account for >75% of the biopesticide market share, biopesticides represent only 5% of the total crop protection market (Olson, 2015). Factors that may limit efficacy and higher adoption of bacterial bioinsecticides include short persistence and low residual activity, product variability, and poor control of tunneling and root-feeding pests. Diverse types of formulations have been developed to optimize delivery against specific insect pests, with efforts concentrating on protection from environmental degradation by the use of melanin as a UV protectant (Saxena et al., 2002; Zhang et al., 2008) and protective delivery systems (Elcin, 1995a,b; Bashir et al., 2016). Formulating two biocontrol agents together, such as *Btk* and blastospores of the fungus *Beauveria bassiana* ((Bals.-Criv.) Vuill.) has been shown to increase efficacy, viability and field persistence (Sayed and Behle, 2017). Due to their sensitivity to environmental conditions during storage and after application, the efficacy of nonspore-forming bacteria will depend on formulation. *Serratia entomophila* is applied to the soil in either liquid or solid granule formulations (Jackson et al., 2018).

The efficacy of formulated *B. thuringiensis*, representing >75% of all biopesticides used (Olson, 2015), has been demonstrated during more than 70 years of commercialization in a variety of agricultural and environmental habitats against multiple target species. *Btk* and *Bt*a are applied in broad-scale agriculture and horticulture and have a particular role in chemical resistance and residue management. In the case of mosquito control, biopesticides based on *Bti* have demonstrated their efficacy in controlling diverse target species, with a concomitant lack of field-evolved resistance. In comparison to *Bti*, *L. sphaericus* formulations are more persistent in rich organic and other habitats due to their recycling and slower settling. However, *L. sphaericus* pesticides have a narrower host range and are more prone to the development of field resistance. Consequently, the relative efficacy of mosquitocidal products based on either bacteria depends on several biotic and abiotic factors, including larval density and feeding behavior, rate of ingestion, and environmental conditions (Lacey, 2007).

12.4 Culture selection and maintenance

After a bacterial isolate has been selected for a pest problem and a commercial market, large scale production can begin, and the following account is based on experiences of large scale (greater than 30,000 L) production of *B. thuringiensis*. The culture selected for industrialization must meet several important commercial criteria. These criteria include the insecticidal spectrum, potency per unit volume of fermentation broth, fermentation media requirements, ease of production, genetic stability, and storage stability (Couch and Jurat-Fuentes, 2014). Once a bacterial isolate has been selected for a pest problem and a commercial market, large scale production begins with the preparation of a stock solution. The bacterial isolate to be produced is sourced from a culture collection and deemed pure. However, a series of tests should be made to confirm purity and prepare working cultures. For *B. thuringiensis*, the parental stock is produced in shake flask cultures grown at 30°C in Lab-Lemco Broth or Tryptic Soy Broth for 48−72 hours (Lisansky et al., 1993). The culture may also be grown in an inoculation medium similar to the actual production medium. Purity checks are made by dilution plating the parent stock on agar plates and incubating these at 30°C for 5 days to ensure the absence of contaminating bacteria and other pollutants or bacteriophages. Aliquots of the broth from the parental culture are also tested for the presence or absence of beta exotoxin if the bacterium is a *B. thuringiensis* isolate. This determination may be made using chromatographic detection (Hernández et al., 2001) or bioassays (Mac Innes and Bouwer, 2009). Generally, once determined to have met all the purity parameters the working cultures are stored in lyophilized vials or in liquid nitrogen refrigeration vials. Usually, batches of 100 vials are made from the parent culture. Whenever new batches of the parental stock culture are made, the starting culture must undergo testing to ensure purity and insecticidal activity at levels equivalent to the reference isolate. This repeated testing is extremely important to ensure product integrity and potency. The number of transfers from the parent culture should be limited to prevent contamination and the loss or exchange of plasmids during subculturing.

12.5 Inoculum preparation

Production of entomopathogenic bacteria usually involves a two-step, single-batch production process. A preculture (inoculum) of the selected bacterium is produced in a volume 1%−5% of the final fermentation. The actively growing inoculum is added to a sterile medium in the main fermenter to be produced in optimal conditions in the main fermenter for maximum growth before harvesting. The preparation of the inoculum is the most important step in the commercial process. If not prepared properly, it will affect the yield of the main fermenter.

As previously described (Beegle et al., 1991; Couch and Jurat-Fuentes, 2014), one or several of the stored vials are aseptically transferred to several 2-L flasks containing tryptic phosphate broth for *B. thuringiensis* or nutrient broth supplemented with 0.05% yeast extract for *L. sphaericus*. The flasks are then placed on a standard rotary shaker and cultivated at 28°C for 24 hours ensuring the bacteria enter the log growth phase. Flasks are checked by microscopic examination to confirm purity before inoculating the seed tank. The volume of the inoculum in the seed tank should be between 1% and 5% of the size of the commercial fermenter (Couch, 2000).

The fermentation medium in the seed fermenter is generally similar to that used in the main fermentation tank (Table 12.1). When thoroughly mixed, the media is sterilized resulting in a milky or creamy color. If the medium appears to be brown this usually indicates that the glucose has caramelized because of overheating and thus is not conducive to bacterial growth and will have to be remade. The procedure for handling the seed fermenter varies by manufacturer. In a typical process for a *B. thuringiensis* seed tank, the inoculum is fermented for no more than 12 hours and is normally used within 8−10 hours under the following conditions: dissolved oxygen (DO) is kept above 20%, fermentation temperature is 30°C−32°C, and pH 5.5−8.0 (pH adjustments are not normally required). If pH > 8 is reached,

TABLE 12.1 An example of inoculation (seed) medium components.

Component	Concentration (g/L)
Soy flour	25.0–35
Corn steep solids	12.5
Glucose	15.0–35
Yeast extract	2.0
NZ amine B	2.0
KH_2PO_4	0.05
Adjust pH to 6.8–7.2	

the inoculum should be rejected. It is a general rule for commercial, large scale bacterial production that the seed culture should be in the log growth phase and actively dividing at the time of transfer to the main fermenter.

12.6 Fermentation

Like other microbes, entomopathogenic bacteria require carbon and nitrogen sources complemented with mineral salts for growth. Commercial production of spore-forming entomopathogenic bacteria involves culture vegetative growth, through transition to sporulation, when each cell produces an endospore and a complement of soluble and/or crystalline insecticidal proteins. Two main methods have been reported for the growth of *B. thuringiensis* and *L. sphaericus* cultures during the production of biopesticides: submerged (liquid), and solid-state fermentation. Since raw materials may comprise 30%–40% of the overall production costs (Lisansky et al., 1993), relevant efforts have focused in identifying cost-effective raw materials supporting adequate growth yields while maintaining high insecticidal activity (Morris et al., 1997), especially in developing countries. The linear relationship between insecticidal activity and Cry toxin concentrations in *Btk* HD-1 grown in different media indicates that toxin concentration can be used as an indicator during the fermentation process and as a predictor of activity (Vu et al., 2012). A number of reports have presented the production of *B. thuringiensis* and *L. sphaericus* biopesticides using wastewater or alternative industrial or agricultural waste derivatives (Tirado Montiel et al., 1998; El-Bendary, 2006). The use of wastewater from the starch industry is feasible for industrial scale production of *Btk* (Vu et al., 2012). Wastewater sludge was shown to support growth of diverse *B. thuringiensis* serovars yielding lower cell counts but higher entomotoxicity per spore compared to synthetic media (Yan et al., 2007). However, both cell counts and entomotoxicity in wastewater sludge systems have been shown to be affected by sludge pretreatment (Tirado Montiel et al., 2001), the amounts of sludge solids (Brar et al., 2009), addition of adjuvants (Brar et al., 2006b), and culture recovery process (Brar et al., 2006a). Since *L. sphaericus* lacks biochemical pathways to use sugars as fermentation metabolites, partially hydrolyzed proteins are used as the protein source. Production of *L. sphaericus* biopesticides using diverse local raw materials from Ghana was reported to support bacterial growth and mosquito larvicidal activity to similar levels detected in pesticides produced using a synthetic medium (Ampofo, 1995). However, these raw materials have several disadvantages that prevent their common use in high-scale commercial production of bacterial biopesticides, including pretreatment costs, variability in composition, seasonality, and local availability. Lower waste output and capital investments have increased interest in solid-state fermentation as alternative production method for bacterial biopesticides. While these methods generally require more intensive labor, they are expected to facilitate the use of bacterial biopesticides in developing countries with low labor costs (Devi et al., 2005). Despite the potential applications of these pilot-plant settings and alternative raw materials that may be of interest to local and regional markets in developing countries, our intent in this chapter is to concentrate on those techniques that are used in the actual commercial production of bacterial bioinsecticides. For more information on the production of entomopathogenic bacteria appropriate to less developed countries, the reader is referred to Chapter 16 in this volume.

In large-scale production, media costs must be balanced with their effect on insecticidal activity. A low-cost medium resulting in low potency will be an inefficient while, conversely, a higher cost medium resulting in a higher density product will be a more efficient use of the costly fermenter infrastructure and operation. Commercial producers of

biopesticides must balance these factors. A list of commonly used ingredients for large scale liquid fermentation of *B. thuringiensis* and *L. sphaericus* is presented in Table 12.2.

Development of the commercial medium is accomplished by setting up a series of experiments using small fermenters to select the optimum concentrations of carbon and nitrogen sources and the appropriate trace minerals. The balance of the carbon and nitrogen concentrations is extremely important. Usually, the results obtained from 150 to −500 L working volume pilot tanks will directly scale up to commercial production. Commercial fermentations of *B. thuringiensis* or *L. sphaericus* will usually be in a fermentation batch of more than 30,000 L, as anything smaller is considered a pilot plant and the process described below would not be cost-effective (Couch, 2000). Fermentation runs in

TABLE 12.2 A listing of some typical commercial fermentation ingredients and their concentration.

Ingredients concentration (g/L)	
Carbon Sources	
Glucose	10–30
Corn syrup DE 95	20–45
Molasses	1.0–18.6
Glycerol	2.0–10
Corn starch	10–15
Nitrogen Sources	
Soy flour	20–40
Cotton seed flour	14–30
Potato protein	15–40
Fish meal	15–20
Peptone	2.0–5.0
Corn steep liquor or solids	15–30
Yeast extract	2.0
Trace Minerals	
$KH2PO_4$	1.0
$K2HPO_4$	1.0
$FESO_4$	0.02
$FESO_4.7H_2O$	0.0005–0.02
$MgSO_4.7H_2O$	0.3
$MnSO_4.H_2O$	0.02
$ZnSO_4.7H_2O$	0.02
$(NH_4)2SO_4$	2.0
$CuSO_4.5H_2O$	0.005
$CaCO_3$	1.0–1.5
PPG 2000	2.0–5.0
Silicone Antifoam	0.1–0.2

Note: Ensure through testing that the silicone antifoam does not interfere with the normal growth of the bacterium and its insecticidal activity.
Source: Adapted from Couch, T. L. (2000). Industrial fermentation and formulation of entomopathogenic bacteria. In "Entomopathogenic Bacteria: from Laboratory to Field Application" (J. F. Charles, A. Delecluse and C. Nielsen Leroux, Ed.), 297–314. Kluwer Academic Publishers, Dordrecht, Netherlands and Lisansky, S. G., Quinlan, R. and Tassoni, G. (1993). "The Bacillus thuringiensis Production Handbook." CPL Press, Newbury, UK.

TABLE 12.3 An example of a commercial fermenter media composition.

Components	Concentration (g/L)
Soy flour	35.0−45.0
Corn steep solids	15.0−20.0
Glucose (DE 95)	10.0−20.0
Yeast extract	0.5
NZ amine B	0.1
$KH2PO_4$	0.8
$MgSO_4$	0.01
$ZnSO_4$	0.01
$FeSO_4$	0.01
PPG 2000 antifoam	2.5

the small tanks are thoroughly analyzed for insecticidal activity, cycle length, and the yield of the active ingredient per unit volume of the fermentation medium. Effects of temperature, cycle length, agitation, pH, DO, and concentration of glucose (*B. thuringiensis*) are constantly monitored.

After a medium is selected (a typical fermentation medium is presented in Table 12.3), at least five consecutive fermentation cycles using this medium are run in the pilot plant tanks. If results are repeatable, the medium will be adopted for commercial production.

A typical commercial fermentation of *B. thuringiensis* is described as follows. The fermenter is charged and the medium sterilized. When cool, the pH is adjusted to 7.2 and the medium inoculated. The sterilized medium can be held until the contents of the inoculation tank meet all transfer criteria. The total fermenter volume will reach 80% of its rated capacity. Running conditions include maximum aeration to ensure thorough oxygenation of the medium (air feed 0.1−1.0 VVM) as confirmed by DO measurements. The pH should be maintained between 6.8 and 7.2, and the glucose feed should begin 1 h after the initiation of fermentation to maintain the glucose level above 2.0 g/L. The sterilized glucose feed consists of 900−1400 kg of glucose in a total volume of 11,000 L. The feed rate usually stabilizes around 7−19 L/minute, and it is stopped when the culture has reached its maximum oxygen utilization rate (OUR) and the log growth phase is over (8−10 hours).

Some fermenters are not set up for automatic pH control and therefore, the media used in these fermenters typically contain a buffer. There is some evidence (Smith, 1982; Yousten and Wallis, 1987) that lack of pH control of *B. thuringiensis* and *L. sphaericus* cultures resulted in a higher potency of the endotoxins when compared to a process in which the pH was tightly controlled. However, most production facilities currently control pH.

The process described above is a fed-batch system in which the carbon source is fed throughout the fermentation cycle until the culture meets its growth peak as confirmed by pH and OUR. The optimum temperature for the commercial process would have been identified in the pilot plant experiments and is usually between 28°C and 32°C. Cooler temperatures extend the fermentation cycle and add to the factory costs, while the cultures do not grow well at temperatures above 32°C.

The fermentation process takes 28−32 hours and is terminated when >80%−90% of the culture has lysed as determined by microscopic examination; at this point, the recovery process is initiated (see below). In *B. thuringiensis* cultures endospore and crystal formation can be observed after 18 hours. The final whole culture (FWC) is cooled to 4°C and the pH is then adjusted to pH 4.5. Complete lysis of the culture is accomplished during cooling and only free spores and crystals will be present in the FWC. The entire cycle from the seed tank to the point just before recovery of the active ingredient is typically between 62 and 92 hours (Couch, 2000).

A typical batch fermentation for the Gram-negative *S. entomophila* was described (Visnovsky et al., 2008) and has been applied at 10,000 L scale (Ehlers, 2007). The fermentation process shows similarities but some important differences to the Gram-positive fermentations described above. Production of *S. entomophila* occurs in a sucrose rich medium (Table 12.4) containing sucrose as a preferred carbon source and yeast extract as a key component to increase cell yield (Visnovsky et al., 2008). Fermentation is carried out at 30°C, aerated to maintain DO levels >20% with

TABLE 12.4 Batch mode fermentation media for *S. entomophila* (Visnovsky et al., 2008).

Components	Concentration (g/L)
Sucrose	40
Urea	2
Granulated yeast extract	10
NH_4NO_3	0.68
KCl	0.46
Na_2HPO_4	0.86

medium maintained at pH 6.5. No benefit has been gained from feeding the culture during fermentation using a fed-batch approach. Unlike *B. thuringiensis*, there is no sporulation to indicate completion of fermentation, so it is important to monitor the fermentation and continue it well into the stationary phase as cells harvested in the log and early stationary phases showed poor survival in storage. Fermentation is completed more rapidly than that of *B. thuringiensis* and produces a cell yield of $>5 \times 10^{10}$ CFU/mL corresponding to >30 g/L dry cell weight, which can be harvested at 26−28 hours from the start of the fermentation. Maintaining high throughput of air and constant agitation for the oxygen demanding *S. entomophila* fermentation can lead to the production of large quantities of foam requiring an effective antifoam delivery system. Bacteriophages can be a threat to production but have been overcome through the use of phage resistant strains in production (O'Callaghan et al., 1992).

12.7 Recovery and concentration steps

Once the fermentation process is completed and the fermenter cooled, the contents must be harvested for further processing of the biopesticide. During the recovery process, the FWC containing culture solids is concentrated from the total culture sludge to obtain the final culture containing the active ingredients. Three techniques can be used in this process: centrifugation, microfiltration and/or evaporation, either individually or in combination, depending on the strain of *B. thuringiensis* or *L. sphaericus* involved. The recovery process generating the final culture must be efficient and recover at least 80% of the FWC solids. The final culture for *B. thuringiensis* and *L. sphaericus* generally contains 6%−8% solids of which 1%−3% will be spores and delta-endotoxin and the remainder of the solids will be soluble and insoluble carbohydrates and proteins (Couch, 2000).

Continuous centrifugation at $>8000 \times g$ is the most prevalent method for active ingredient recovery from the *L. sphaericus* FWC, and is also the least expensive. Lisansky et al. (1993) defined the basic parameters for centrifugation recovery conditions. The starting solid content of the FWC should be 4%−6%. Prior to centrifugation a flocculant may be used. Also, lowering the pH to 4.5 seems to aid in the recovery of the solids. The final concentration following centrifugation will range between 15%−30% solids. Lisansky et al. (1993) state that if a starting FWC has a lower solid concentration, centrifugation may be inefficient, since the recovery time of this method is longer and more costly. It is at this point that combining centrifugation with evaporation may be a viable option. Centrifugation can also be made more efficient by diluting the FWC before centrifugation to remove more of the dissolved solids. If the FWC is viscous, enzyme treatment may also improve recovery efficiency. The average loss of the active ingredient through the centrifugation step is typically 10%−15%.

Microfiltration is a method that removes the active ingredients from the FWC in a very efficient manner. Almost 95%−100% recovery of the active solids is possible. This is particularly true for *B. thuringiensis*, because of the small size of the delta-endotoxin. The filters can be membrane or ceramic and pore size of 0.1 to 0.2 microns is the preferred size. During recovery, the FWC is continuously washed and cooled to optimize efficiency and potency. This method has the additional benefit of removing any undesirable metabolites. The downside of microfiltration is that it is very expensive, although its efficiency often compensates for the expense of the purchase and operation of the system.

The need for cell concentration will depend on the cell density in the FWC and the concentration needed for the final product. If high cell density can be achieved in the fermenter and the fermentation byproducts are not detrimental to the cells, the FWC becomes the final culture and product. This is the case for *S. entomophila* (see below). Every

commercial company has its own recovery procedures which generally are not shared. It is safe to assume commercial companies exercise extreme care during the recovery step to preserve the insecticidal activity of the entomopathogenic bacteria. High temperature and shear will destroy activity quickly and must always be avoided. The recovery process must also be structured to prevent exogenous microbial contamination of the recovered concentrate.

12.8 Formulation

The formulation of the recovered final culture powder or liquid concentrate is the final important step in the preparation of a commercial product of any entomopathogenic bacterium. The formulation must be made from readily available inert ingredients which have the important function of maintaining viability of the organism through storage and distribution and facilitating the application of the bacterium through standard application systems. The insecticidal activity of the formulation must be stable under normal storage conditions found in typical warehouses. The shelf-life expectation for these formulations is 18−36 months when stored in a cool dry place below 30°C. Prolonged storage at higher temperatures is discouraged and will have an adverse effect on the activity of the formulation and its performance in the field. The commercial formulation constitutes the form and contents of the bioinsecticide as supplied by the manufacturer to the distributor and ultimately to the end-user (Couch and Ignoffo, 1981). Formulations of bacteria are exactly like those needed for chemical pesticides. The latter has been in the market longer than bacterial biopesticides and most equipment has been developed for these products. Where possible, formulations of entomopathogenic bacteria should be adaptable to this equipment. The formulation must be optimized for the distribution of the entomopathogenic bacterium on the target crop or other site depending on the strain of the bacterium and target insect. In the case of aquatic sites, a dispersant is often used in liquid *Bti* to facilitate the dispersion of the delta-endotoxin through the feeding horizon of the mosquitoes or blackflies. In agricultural applications, it is not only important to develop a formulation that is easily mixed and applied, but it should also have appropriate ingredients to spread the formulation on the target surface and provide sticking properties and some UV protection to the bacteria. Diverse micro/nanotechnology applications have been used to encapsulate *Bt* and its insecticidal proteins to overcome formulation instability and UV degradation of pesticidal proteins (De Oliveira et al., 2021).

Formulations for bacterial biopesticides can be categorized as liquid or dry products (Burges and Jones, 1998). Liquid products, suspension or emulsifiable concentrates, are incorporated into a tank mix with a diluent before application. Some dry formulations, wettable powders and dispersible granules, will also be applied through a tank mix with a diluent, but dry granules, pellets, briquettes and donut formulations are applied directly. Aqueous liquid formulations are the least expensive to produce and formulate. The final culture represented by the concentrated fermentation slurry is the base for these formulations. The final culture is stabilized using appropriate bacteriostatic and fungistatic agents to prevent secondary fermentation. Also included are usually a dispersant, suspending agent, and sometimes a thickening agent when the final culture has a very low viscosity. The exact formula for the final culture is usually a closely guarded company secret and is listed only in the confidential statement of formula for each aqueous suspension. Several articles present extensive lists of typical liquid and solid formulation components (Couch and Ignoffo, 1981; Beegle et al., 1991; Lisansky et al., 1993; Burges and Jones, 1998; Couch, 2000). For liquids, these lists include the most commonly used dispersants, suspending agents, fungistats, buffers, stabilizers, preservatives, thickeners and antievaporation agents. Aqueous liquid formulations have very strict storage requirements since they are more susceptible to raised temperatures and exogenous contamination once the containers have been opened. Storage temperature must not exceed 30°C and storage at 10°C−15°C is preferred. Generally, when stored properly aqueous liquid formulations will work at label rates for two seasons. The potency of the product may decline over time but the recommended rates on the label are usually adjusted to consider a 10%−15% decrease in label potency through storage.

The final culture can be spray dried to produce a dry technical grade active ingredient (TGAI), which is the base for dry formulations. Care must be taken as the drying process can destroy the insecticidal activity of the TGAI. Experimentation on a lab-scale drier is used to select the inlet and outlet temperature for the commercial drier. The handling of the final culture during drying and the powder after drying is very important. The inlet and outlet temperatures on the spray drier must be continually monitored to maintain the TGAI below the critical temperature which is destructive to the insecticidal activity. Once dried, the powder is recovered by collecting it with cool dehumidified air. Since the powder is an insulator, it will retain heat for a long time in the drums into which it is packed for storage and sale, so it must be less than 30°C when packed. Each company has its own methodology, with drying and recovery conditions proprietary. If done properly an optimized spray drier can recover 85%−95% of the active ingredient from the final culture. The spray-dried powder is easily stored and very stable.

The spray-dried powder (TGAI) is then mixed with diluents and may be compressed or molded to make a solid formulation. The types and kinds of diluents are extensive and reported in Burges and Jones (1998). Initially the most common of the dry formulations was the wettable powder, which contains a diluent (usually neutral clay) and appropriate surfactants and dispersants to improve handling in the field. These inerts should be compounded to optimize product deposition to ensure performance against the target insect. The powders also included dust formulations, which are rarely used today. The most common and preferred dry formulation of entomopathogenic bacteria is the water-dispersible granule (WDG) because, in contrast to the wettable powder, they are not dusty and mix almost instantly. These are designated WG, WDG or dry flowable. Like the powders, the WDG has an excellent shelf life with no noticeable decrease in activity when stored for up to 3 years in a cool dry environment. The WDG formulations contain many of the same ingredients as the wettable powder but they are granulated in a pan granulator or fluid bed drier. Couch (2000) prepared a comprehensive list of the specific types of dry diluents, carriers, and surfactants used in this type of formulation.

Liquid, wettable powder and WDG formulation types have been used for *Btk*, *Bti*, and *L. sphaericus* for incorporation into tank mixes for spraying. However, *Bti* and *L. sphaericus* have been applied in several unique formulations which are applied directly to water. Specifically, these are unique granules, briquettes and a donut formulation. Both *Bti* and *L. sphaericus* are formulated on corn cob granules of various mesh sizes. The most common mesh sizes are 10/14 and 5/8. The *Bti* and *L. sphaericus* TGAI are formulated on the corn cob carrier with an edible mineral oil or soybean oil binder. The granules, depending on the concentration of TGAI formulated onto the corn cob, are applied using aerial and ground equipment at concentrations of 1−9 kg/ha. The granules are capable to penetrate heavy cover (trees, bushes, grass, crops, etc.) which prevent the penetration of an aqueous liquid spray. They are applied dry with no water mixing required. *Bti* can also be formulated on-site with sand and an oil binder to provide a dense granule with even better penetration of the heavy cover. For special applications, the *L. sphaericus* granule can be packed into water-soluble pouches for application to containers, stormwater catchment basins, etc. Briquettes have been formulated to provide prolonged residual control and some formulations last up to 180 days in standing water, including abandoned swimming pools, cisterns, open ditches, sewage ponds, water tanks, and other standing water sites. A donut-shaped combination briquette using both *Bti* and *L. sphaericus* as its active ingredients has been developed and is patented by the manufacturer.

Gram-negative bacteria lacking resistant spores are much more sensitive to postproduction environmental conditions than spore-formers and the difficulty of maintaining viability from production to use has limited the development of this group of organisms. Marketing may have to adapt to the characteristics of the organism. With the improvement of fermentation strategy and conditions for *S. entomophila* described above, a final culture was produced which could be stored under refrigeration for more than 6 months without significant losses. This provided enough stability for a product to be manufactured and distributed through cool store networks. This 6-month period was sufficient to organize sales and treatment of a seasonal pest like the New Zealand grass grub (Jackson et al., 1992), but the need for cool temperature storage limited the distribution of the product. To overcome this limitation pellet and granule formulations were developed utilizing a bacteria/biopolymer mix which maintained bacterial survival for up to a year in ambient conditions (Jackson, 2017). Biopesticides based on Gram-negative *C. subtsugae* or *B. rinojensis* contain nonlive bacteria, spent fermentation media and formulating ingredients in a wettable powder or liquid format which is stable if stored in cool conditions. These examples show that live Gram-negative bacteria or their culture products can be incorporated into stable products for commercialization. They are not as robust as the dry spore/toxin products based on spore-forming Gram-positives, but with careful management of the market chain they can provide useful solutions to otherwise intractable pests.

12.9 Formulation standardization

Once a formulation is selected it must be standardized to ensure there is no variation in the concentration of the active ingredient or the potency of the formulation. Standard Operating Procedures include determination of color, odor, particle size, specific gravity/density, viscosity (liquids only), particle size, wetting times (powders and dispersible granules), pH, and suspensibility. Each lot of the finished formulation produced is checked for conformance to the above parameters. If the batch has any of the parameters which are not the adopted standard for the formulation it is rejected and sent for reformulation and recovery.

12.10 Quality assurance methods

Quality during the production process is monitored using guidelines for Good Manufacturing Practice and Standard Operating Procedures for each step of the process to ensure a consistent end product. Bacterial insecticides contain live

bacteria and/or bacterial products and so specific quality control methods must be implemented. When entomopathogenic bacteria were first developed as biopesticides there were no chemical or reliable biochemical methods to determine the insecticidal potency of the TGAI and formulated products. Insect bioassays were used to standardize these products. An initially proposed method (Dulmage et al., 1971) was later standardized for *Btk* isolates (Beegle et al., 1986). For *Btt*, a method of standardization was described using Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] larvae (Riethmüller and Langenbruch, 1989). Quality assurance of products containing live Gram-negative bacteria, such as *S. entomophila*, is assured by tests for purity of the strain and virulence of the starter culture followed by viable cell quantification, purity and virulence testing of the final product. Products based on transconjugate bacteria, for example, bacteria producing toxins from more than one *B. thuringiensis* serotype, are difficult to standardize using insect bioassays. In this case, ELISA and HPLC methods are frequently employed as a proxy to measure potency. Similarly, a proteomic method was presented to accurately determine the toxin composition and relative amounts of insecticidal proteins in *Btk*, *Bt*a, *Bti*, and *Btt* biopesticides (Caballero et al., 2020). However, all these methods will give a positive result for the presence of the insecticidal protein even if it has been denatured or no longer possesses insecticidal activity. This is the primary reason why current commercial products using entomopathogenic bacteria still use insect bioassay techniques for standardization.

As the mosquito biolarvicides *Bti* and *L. sphaericus* are used in environmentally sensitive areas including drinking water catchments, the World Health Organization convened a panel to develop a set of guidelines to assure safety (World Health Organization, 1999). Although these guidelines (WHOPES) were developed for entomopathogenic bacteria used in public health, they can also be applied to agricultural isolates of *B. thuringiensis*. In fact, these guidelines are used as the basis for many of the quality control parameters for the final formulations of commercial entomopathogenic bacteria. A common presence through all the bioassay methodologies and guidelines is the need to conduct all bioassays of the commercial formulations against an internal standard of known potency. The bioassay methods cited, describe these standards and how they are maintained. After the potency determinations have been completed a mouse safety test must be run on the TGAI used in the commercial formulations. Five mice are injected with $10e^6$ spores subcutaneously and observed for 7 days. The test was designed to screen for infectious or exogenous contaminants. Although not required by the regulatory authorities, every batch of TGAI and the formulated product are also screened as per the WHOPES guidelines. The exogenous microbial contaminant report must show that the following contaminants do not exceed WHOPES limits: coliforms <10/g; *Staphylococcus aureus* Rosenbach absent in 1 g; *Salmonella* spp. absent in 10 g; *Enterococcus* (*Streptococcus*) *faecalis* Andrews and Horder (Schleifer and Kilpper-Bolz) 1 X 10^4/g; viable yeasts and molds <100/g and no human pathogens. Once the products meet the physical chemistry standard, potency standard, and exogenous contaminant standard the products are packaged and cleared for sale.

12.11 Conclusion

The commercial fermentation methodologies used for the production of entomopathogenic bacteria currently on the market are highly evolved and optimized to produce the maximum insecticidal activity per unit volume of fermentation media. However, manufacturers continually strive to improve the yields while lowering manufacturing costs and increasing shelf life and environmental stability to ensure the products can compete in the marketplace. Advances have been made to increase the residual activity of the formulations, including extended-release properties. Increased shelf life and residual activity are especially critical to the success of nonspore forming entomopathogenic bacteria. Current products based on Gram-negative bacteria can include live or dead cells, in addition to insecticidal metabolites and byproducts. Advancements in formulation and delivery, especially in cases of products with live bacteria, will increase the chances for successful commercialization of Gram-negative bacterial entomopathogens.

Since most commercialized bacteria are natural isolates and not trans-conjugates or recombinant strains, they are considered “green formulations” and are used in organic farming. This reliance on natural isolates fuels interest in prospecting for new and more active isolates.

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Chapter 13

Production of entomopathogenic viruses

Steven Reid[1], Henry de Malmanche[1], Leslie Chan[2], Holly Popham[3] and Monique M. van Oers[4]

[1]School of Chemistry & Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia, [2]Thermo Fisher Scientific, Brisbane, QLD, Australia, [3]AgBiTech, Columbia, MO, United States, [4]Laboratory of Virology, Wageningen University and Research, Wageningen, the Netherlands

13.1 Introduction

13.1.1 General introduction

Most documents addressing the topic of the commercial production of entomopathogenic viruses will concentrate on baculoviruses due to broad knowledge of these viruses and the commercial products available. This chapter is no exception and will concentrate on the production of baculoviruses in vitro using insect cell culture technology in comparison to in vivo production in insects. The point of view taken by this chapter is that wild type baculoviruses have an important role to play in the control of insect pests but a major limitation to their wider use is the lack of a cost-effective in vitro production technology. This point has been well made by a number of previous authors over the past 30 years (Weiss and Vaughn, 1986; Murhammer, 1996; Black et al., 1997; Ravensberg, 2011b). However, while the topic of in vitro production of baculoviruses has been covered in general terms, the specific yield targets and issues still to be addressed for this to become a reality has been poorly addressed and this chapter attempts to address that deficiency in the literature. Following a brief introduction of entomopathogenic viruses and an explanation of why baculoviruses have received the most attention, the status of in vivo production of baculoviruses will be described and the point made that some companies have successfully developed products using this mode of manufacturing for baculoviruses (Section 13.2). The current status of in vitro production of baculoviruses and the limitations to its successful application in the market, along with suggestions for further research will then be addressed in detail (Sections 13.3–13.5). Finally, some comments will be made as to what needs to happen for in vitro production of baculoviruses to be realized (Section 13.6).

This chapter does not address the many issues of product formulation to help protect the virus from ultraviolet (UV) damage and to allow better sticking and spreading of the virus on various plant surfaces or farmer education requirements to encourage the use of Integrated Pest Management strategies, all of which are required to make baculovirus biopesticides a success. These topics have been covered in other publications, and companies currently selling baculovirus products produced in vivo have successfully addressed many of these issues (Payne, 1982; Black et al., 1997; Ravensberg, 2011b; Harrison and Hoover, 2012). While in vitro production of baculoviruses faces many challenges, the sterile harvest of the final product is not one of them, and the final concentrated harvest from such processes can readily take advantage of the formulation and application technologies already developed for the in vivo-produced virus products.

13.1.2 Entomopathogenic viruses

Many viruses have been shown to infect insects and these include both DNA and RNA viruses. It is beyond the scope of this chapter to review them and the reader is referred to other references for detailed discussions of the full range of entomopathogenic viruses (Payne, 1982; Miller and Ball, 1988; Asgari and Johnson, 2010; Harrison and Hoover, 2012; Ryabov, 2017; Tijssen et al., 2017; Williams et al., 2017; Sosa-Gómez et al., 2020). What is clear from all reviews of insect viruses is that baculoviruses have received more attention as pest control agents than any of the other groups because they have never been found to cause disease in any organism outside the class INSECTA and they are

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responsible for most of the natural viral epizootics observed in lepidopteran insects and sawflies (Payne, 1982; Miller, 1997; Moreau and Lucarotti, 2007).

Baculoviruses are DNA viruses that are occluded in protective protein capsules called occlusion bodies (OBs), which allow them to persist for long periods outside the host, and such occlusions protect the virions from damage when applied to crops using traditional spray equipment. A particular paper (Harrison and Hoover, 2012) provided an overview of baculoviruses and other occluded insect viruses, pointing out that occlusion of virions has been observed predominately in viruses of insects. Two other groups of occluded insect viruses are the entomopoxviruses (EPVs; family *Poxviridae*, subfamily *Entomopoxvirinae*) and the cypoviruses (family *Reoviridae*, subfamily *Spinareovirinae*, genus *Cipovirus)*. The other viruses in the family *Poxviridae* and *Rheoviridae* are nonoccluded, hinting to particular importance of occlusion for these insect-infecting viruses that may be related to the transient nature of many insect populations. Also in the family *Nudiviridiae* (Harrison et al., 2018) which mainly contains nonoccluded DNA viruses, a few exceptions of arthropod-infecting viruses that do form occlusions are found (Bezier et al., 2017; Yang et al., 2014). While also some EPVs and cypoviruses have been assessed as potential biopesticides, the vast majority of viruses registered for use as biopesticides are baculoviruses that target either lepidopteran pest insects (Kabaluk et al., 2010) or forest damaging sawflies (Moreau and Lucarotti, 2007; Hajek and van Frankenhuyzen, 2017).

13.1.3 Baculoviruses

13.1.3.1 Taxonomy

Viruses categorized in the family *Baculoviridae* are rod-shaped, double-stranded DNA viruses with a large circular genome varying in size between 80 and 180 kbp (Harrison et al., 2018). The family is divided into four genera: *Alphabaculovirus* [containing nucleopolyhedroviruses (NPVs) that infect lepidopteran insects], *Betabaculovirus* [containing the granuloviruses (GVs) found in lepidopteran insects], *Gammabaculovirus* (NPVs infecting hymenoperan insects), and *Deltabaculovirus* (NPVs infecting insects in the order Diptera) (Jehle et al., 2006; Herniou et al., 2012). The alphabaculoviruses are taxonomically separated into group I and group II NPVs (Zanotto et al., 1993). The *Autographa californica* multicapsid NPV (AcMNPV) is the type species of the genus *Alphabaculovirus* and belongs to group I. It is extensively used in biotechnology to produce recombinant proteins for vaccines, diagnostics, as well as viral vectors for gene therapy (see, e.g., Urabe et al., 2002; Airenne et al., 2013; van Oers et al., 2015; Possee et al., 2020). AcMNPV has a relatively broad host range and is not used in biocontrol. The group I NPV A*nticarsia gemmatalis* NPV used in soybean crops, is one of the most widely used baculoviruses for biocontrol. *Helicoverpa armigera* (Hear) NPV is used to control the cotton bollworm and belongs to group II. *Cydia pomonella* GV is the type species of the genus *Betabaculovirus* and is successfully used in Europe in apple orchards to control the coddling moth. In this chapter, we will focus on the NPVs and GVs that have commercial potential against lepidopteran insects.

13.1.3.2 Baculovirus phenotypes and their function

Most baculoviruses adopt two genetically identical, but phenotypically different, infectious viral structures (Fig. 13.1) that perform different functions in the infection cycle, see reviews by Slack and Arif (2007), van Oers and Vlak (2007), Rohrmann (2019), and Blissard and Theilmann (2018). The form that is required in the final formulation of biocontrol products needs to infect larvae orally. Hence, the virus must be produced in the form of viral OBs, proteinaceous capsules that each contains many so-called occlusion-derived viruses (ODVs), (with the exception of the betabaculoviruses that contain a single ODV per OB). ODVs contain either one (GVs and single-nucleocapsid (S) NPVs) or multiple nucleocapsids (MNPVs) surrounded by a single envelope (Fig. 13.2).

The OBs are ingested orally by the larval stages of the (pest) insect and fall apart in the insect's alkaline midgut. The released ODVs then infect columnar cells in the midgut epithelium by making use of *per os* infectivity factors (PIFs) (see Section 13.1.3.3). Once inside the cells, the viral nucleocapsids move along actin cables to the nucleus (Ohkawa et al., 2010), where DNA replication is initiated. Meanwhile, viral capsid proteins are being produced that encapsulates the new DNA to form progeny nucleocapsids that move toward the cytoplasm. At the cell surface, these nucleocapsids acquire an envelope by budding through the plasma membrane to form budded viruses (BVs). These BVs spread the infection to the other larval tissues. Details on these processes can be found in Blissard and Theilmann (2018). Most baculoviruses contain in their BV envelope the viral fusion protein F that allows them to bind and enter insect cells (Long et al., 2006; Westenberg et al., 2004; Wang et al., 2016; IJkel et al., 2000; Pearson et al., 2000). However, in the group I NPVs of the genus *Alphabaculovirus*, this function is taken over by the GP64 protein (Blissard

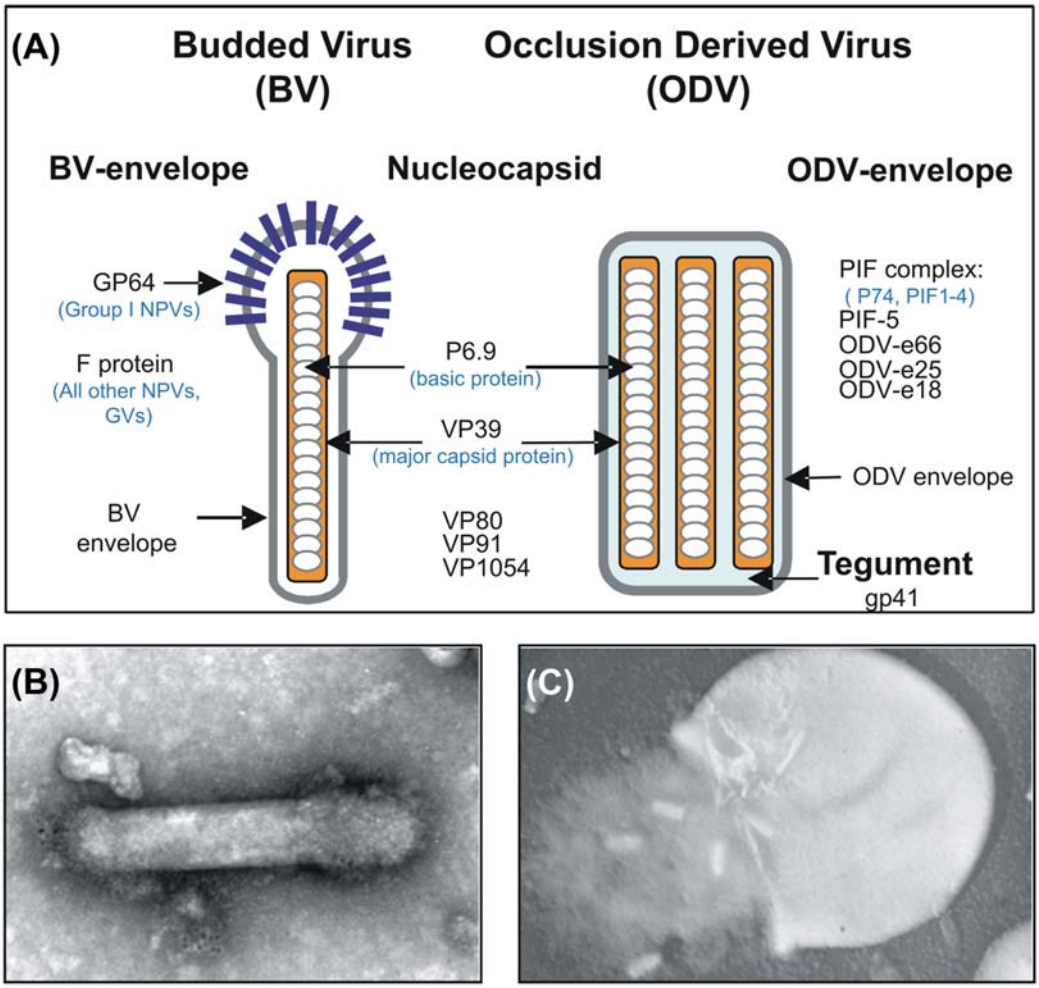

FIGURE 13.1 Baculovirus phenotypes. Schematic (A) of budded viruses (BVs) that spread the infection through the insect body and occlusion-derived viruses (ODVs) that initiate infection in midgut epithelium cells after oral uptake of occlusion bodies (OBs). Important structural proteins present in the BV envelope, the nucleocapsid (orange structure), and the ODV envelope and tegument (light blue), are indicated. GP64 is the BV envelope fusion protein of alphabaculovirus group I NPVs. In all other NPVs and GVs, the F protein serves this function (except for gammabaculoviruses, which do not make BVs). The ODV envelope contains the per os infectivity factors (PIFs), five of which are known to form a complex. PIF are crucial for oral infection. Electromicroscopy shows BV (B) and OB releasing ODVs under alkaline conditions (C). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book) *Modified from van Oers, M. M., Vlak, J. M., 2007. Baculovirus genomics. Curr. Drug. Targets. 8, 1051–1068, Copyright Bentham Science Publishers.*

and Wenz, 1992; Monsma et al., 1996; Oomens and Blissard, 1999). These proteins mediate the pH-dependent fusion of the viral envelope with the membrane of target cells, the first step in BV infection.

In secondary infected cells, the process of infection and virus budding is repeated but is followed at a later stage by the retention of nucleocapsids inside the nucleus. These nucleocapsids obtain an envelope derived from the inner nuclear membrane (Braunagel et al., 2009) and become the new ODVs. These ODVs become occluded in the major OB protein, polyhedrin (NPVs) or granulin (GVs) (Rohrmann, 1986). Internal body cells, and especially fat body cells, are the place where massive amounts of ODVs are made and become occluded in OBs. The OBs are released into the environment when the insect liquefies as a consequence of the infection and serve to horizontally transmit the virus to new individuals.

13.1.3.3 Occlusion-derived virus–midgut interactions

The first step in a baculovirus infection of an insect is mediated by PIFs, a specific set of at least 10 ODV envelope proteins (see Fig. 13.1). PIF proteins are encoded by conserved baculovirus genes and are specifically required for oral infection in the gut. Hence, their presence is a prerequisite for the use of baculoviruses as biocontrol agents and

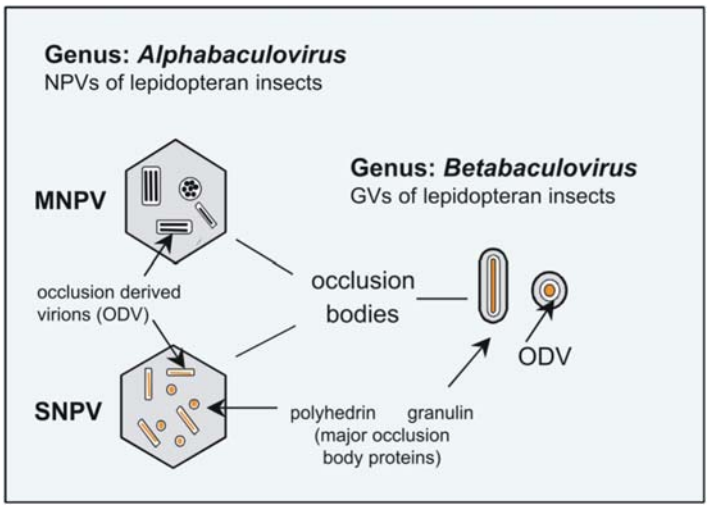

FIGURE 13.2 Baculovirus occlusion body (OB) morphology of lepidopteran-infecting baculoviruses. Comparison of OB morphology in multiple (M) and single (S) nucleocapsid nucleopolyhderoviruses (NPVs; genus *Alphabculovirus*) and granuloviruses (GVs; genus *Betabaculovirus*). *Modified from van Oers, M. M., Vlak, J. M., 2007. Baculovirus genomics. Curr. Drug. Targets. 8, 1051–1068; Copyright Bentham Science Publishers. (For color version of this figure, the reader is referred to the online version of this book).*

therefore they are discussed in a bit more detail in this review. About 10 PIF proteins have been identified, so far (Faulkner et al., 1997; Kikhno et al., 2002; Pijlman et al., 2003a; Ohkawa et al., 2005; Simon et al., 2005a; Fang et al., 2009; Sparks et al., 2011a; Nie et al., 2012; Zhu et al., 2013; Liu et al., 2016; Javed et al., 2017; Boogaard et al., 2019). Most PIF proteins assemble into a complex embedded in the envelope of the ODV particles. PIF1, PIF2, PIF3, and PIF4 form a stable core complex, with which all other components associate (Peng et al., 2010; Peng et al., 2012; Boogaard et al., 2018; Wang et al., 2019). It was recently shown that when this complex cannot be formed due to the deletion of a crucial component (e.g., PIF1) all other PIF proteins are vulnerable to proteolytic decay (Boogaard et al., 2017). PIF proteins appear to be highly species-specific and cannot simply be interchanged without losing oral infectivity (Song et al., 2016), which may be partly due to the interactions with other proteins in the PIF complex, but interactions with cellular factors may also be of importance here.

Although considerable progress has been made in recent years (Boogaard et al., 2018; Wang et al., 2019), we lack an understanding of how all these proteins interact to achieve oral infection. We know that without P74 (PIF0) the ODVs do not bind to midgut cells, and that PIF1, PIF2, and PIF3 are required for cell entry after the binding (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Mu et al., 2014). Without PIF3 fusion of the viral and cellular membranes seems to be incomplete (hemi-fusion status), as could be concluded from combining the results of R18 dequenching assays (Ohkawa et al., 2005) with those of live-imaging of the infection process using ODVs with fluorescently-labeled nucleocapsids (Boogaard et al., 2020). PIF9 is also not needed to bind to isolated midgut epithelial cells, but the nucleocapsids failed to enter the cells (Boogaard et al., 2020). PIF8 (VP91/P95) has a special position, as it has two distinct functions. It is needed for BV and ODV production, while its zinc-finger domain is needed for oral infectivity (Zhu et al., 2013; Javed et al., 2017). PIF5 (ODV-E56) is the only PIF protein that was not found in the PIF complex and it was shown not to be essential for binding or fusion (Sparks et al., 2011a). Disulfide bond formation is crucial for the function of PIF5, which serves as a substrate for the viral sulfhydryl oxidase P33 (Zhang et al., 2020), but its exact role in oral infection remains enigmatic. Another protein that is important for oral infectivity is ODV-E66 (Xiang et al., 2011) and small peptides with homology to this ODV envelope protein blocked AcMNPV infection in *H. virescens* larvae (Sparks et al., 2011b). Furthermore, *Bombyx mori* NPV ODV-E66 was shown to interact with PIF4 (Dong et al., 2014). These data seem to support the renaming of ODV-E66 as a PIF protein, although strictly speaking it is not absolutely required for oral infection.

13.2 In vivo production of baculovirus-based biopesticides

13.2.1 Introduction

Baculoviruses are used for insect control across many agricultural systems including forestry, horticulture and row cropping (Haase et al., 2015; Sosa-Gomez et al., 2020; Sun, 2015). At present there are at least 14 NPV (alpha- and deltabaculovirus) and 10 GV (betabaculovirus) species developed into >80 products worldwide (Grzywacz, 2017; Grzywacz and Moore, 2017). All of these are produced in vivo using cultured insects on an artificial diet.

13.2.2 Increased adoption of nucleopolyhedrovirus products

There has been a surge in the commercial use of baculovirus in the last few years especially in the adoption in field crops as well as specialty crops. The increased need has come from the pressure to replace chemical insecticides and increasing resistance to pesticides which requires a combined strategy of pest management including baculoviruses (Haase et al., 2015).

One of the best-known baculovirus field adoptions is AgMNPV for control of the velvet bean caterpillar, *A. gemmatalis* Hübner, in Brazilian soybean fields (Moscardi et al., 2011). Though AgMNPV is not used to the degree it was previously due to changes in grower cultural practices, it opened the door to mainstream adoption of NPV. Two other recent factors have influenced the increased adoption - the geographical expansion of two key pests and the continued increase in insecticidal resistance.

The Old World cotton bollworm, *H. armigera* (Hübner), is an invasive pest found in 143 countries and resistant to 49 insecticides (Pozebon et al., 2020). It was first found in 2013 in Brazil which spawned an interest in *H. armigera* NPV (HearNPV) as a control measure but could have arrived as early as 2008 (Pozebon et al., 2020; Sosa-Gómez et al., 2020). *H. armigera* is a pest of cotton, soybean, and corn as well as other crops. HearNPV had previously successfully been used in Australia, India, South Africa, and China (Sun, 2015; Grzywacz, 2017). For several seasons *H. armigera* had outbreaks particularly, in the central region of the country, and up to 1.3 million Ha of row crops were treated with HearNPV (Sosa-Gómez, 2017). In recent seasons, *H. armigera* pressure has remained low however interest in baculoviruses has remained.

Because of the encouraging control results provided by HearNPV in Brazil, the use of baculoviruses and other microbial control agents has increased (Sosa-Gómez et al., 2020). Several new products have been registered including products based on *Chrysodeixis includens* NPV (ChinNPV), *A. californica* MNPV (AcMNPV), and *Spodoptera frugiperda* MNPV (SfMNPV) as well as a first combination product combining HearNPV + ChinNPV (Sosa-Gómez et al., 2020). *C. includens* (Walker), the soybean looper, has become more prevalent in cotton and soybeans. *H. armigera*, *C. includens*, and *S. frugiperda* migrate easily between crops due to overlapping crops throughout the year leading to expansion of insect host range as well (Valicente, 2019).

The highly adaptable and polyphagous fall armyworm (FAW), *S. frugiperda* (Smith), has emerged as another invasive lepidopteran. *S. frugiperda* previously localized to the Americas, was controlled largely by genetically modified maize before spreading globally. In 2016, *S. frugiperda* was found in portions of Africa and Asia where it has continued to spread nearly throughout Africa and Asia including nearly all of China where it infests millions of hectares of maize (Hruska, 2019; Lei et al., 2020). In February 2020, it was also found in Australia (Fan et al., 2020). Many growers impacted by the spread of FAW are smallholder maize farmers without access to expensive control technologies and rely on cultural controls for farming (Hruska, 2019). Because of the exceptional damage caused by these larvae, alternative and affordable controls are needed. Many different natural control methods are under investigation including SfMNPV which has been registered in several African countries (Hruska, 2019). SfMNPV products are also being developed in China (Lei et al., 2020).

Baculoviruses were recently formally acknowledged as a new mode of action (Group 31) by the Insecticide Resistance Action Committee (IRAC) which focuses on the preservation of the use of insecticides through effective resistance management to promote sustainable agriculture (Sparks et al., 2020). This inclusion was fueled by the increase in natural products and biologics now used as control options and places these alternatives as a valid control to use with chemical insecticides to manage resistance. SfMNPV has been demonstrated to be effective against strains of *S. frugiperda* resistant to major classes of insecticides and Bts (Bentivenha et al., 2019). ChinNPV has also been shown to be effective on chemical insecticide-resistant strains of *C. includens* (Godoy et al., 2019).

13.2.3 Production using infected larvae

A number of examples of successful programs to produce baculovirus biopesticide products using infected larvae have been documented over the past 40 years (Ignoffo, 1973; Shapiro, 1986; Ignoffo, 1999; Buerger et al., 2007; Moscardi et al., 2011; Harrison and Hoover, 2012, Grzywacz and Moore, 2017). A review of the use and regulation of microbial pesticides worldwide lists a number of baculovirus products listed for use on various crops in China, India, Europe, Argentina, Brazil, Canada, USA, and Australia (Kabaluk et al., 2010). The current in vivo-produced baculoviruses of major economic importance target heliothine larvae (*H. virescens* and *Helicoverpa zea* (Boddie), in North America, and *H. Armigera*, in China, India and Australia), *Anitcarsia gemmatalis* (Hübner), in Brazil and Argentina, *C. pomonella* (Linnaeus) in Europe, Argentina, Canada, USA, and New Zealand and various Spodoptera pests [*Spodoptera litura* (Fabricius), in China, India, and Europe and *Spodoptera exigua* (Hübner), in China, Europe, and USA].

While some manufacturing facilities in the major industrialized nations are technically sophisticated with high levels of automation and quality control, many in the less developed countries are labor-intensive and can suffer from a lack of quality control (Van Beek and Davis, 2016; Grzywacz et al.; 2014). Details of the more sophisticated systems are understandably not publicly available but protocols for more basic systems are published (Van Beek and Davis, 2016).

13.2.4 Challenges for existing baculovirus pesticides and the case for in vitro production

Much of baculovirus research in the last 25 years has centered on fundamental research including sequencing and genetically modifying baculoviruses (Grzywacz, 2017). Additionally, many virulent isolates have been identified for different baculovirus species but there remains the larger challenge to produce a potent product to match demand at a reasonable cost. Studies are needed on commercially relevant pests, potency comparisons with alternative controls, field studies on replicability and reliability, alternative formulations, shelf life, ease of use etc. (Grzywacz and Moore, 2017). A recent new formulation was published using wax microencapsulation to protect baculoviruses from UV degradation for example that shows promise (Wilson et al., 2020). Much of the commercial production knowledge is not shared since it is considered intellectual property.

In very general terms $1 \times 10^{11} - 5 \times 10^{12}$ OB per Ha are required to control a caterpillar pest on a crop and 10^9 to 10^{10} OB can be produced per infected caterpillar (Van Beek and Davis, 2016; Grzywacz et al., 2014). This means 10−5000 infected caterpillars are required to make sufficient virus per Ha treatment.

Many production problems need to be overcome in order to produce baculoviruses effectively in vivo, including optimization of the diet used to ensure a good growth rate of the larvae (Elvira et al., 2010), and effective harvesting of the virus from a complex mixture of insect bodies and diet while keeping the final product reasonably free of contaminants (Buerger et al., 2007). While no doubt many challenges remain for in vivo production of baculoviruses, including ongoing quality assurance issues and high labor costs (in developed countries at least), significant global expertise exists and groups interested in accessing low cost in vivo-produced viruses can find many successful commercial operations in this regard in Europe, the USA, Australia, Brazil, China, and India and possibly elsewhere.

Assuming a global market for wild type baculoviruses exists of 100,000−500,000 Ha for each of the major pests currently targeted by in vivo-produced baculoviruses (HearNPV, AgMNPV, CpGV, PlxyGV, and various *Spodoptera* NPV) at a farm gate price of US$20/Ha, then a case can be made for in vitro production of baculoviruses. Given the fluctuating market potential for any one virus, it is important that a production facility produces multiple viruses. In vitro production would certainly be preferable to in vivo production if a company wanted to produce multiple viruses at scale, as the final production process would be generic, and could be conducted relatively easily in a single facility with a single large bioreactor. Frozen stocks of multiple production cell lines and relatively small stocks of virus would be easier to manage than the maintenance of multiple large volume insect colonies. In vitro production also avoids problems such as the development of diseases in a production colony and contamination of the final product with other microorganisms and potentially allergenic insect parts (Harrison and Hoover, 2012). In addition, should recombinant baculoviruses expressing toxins resulting in faster rates of kill ever be allowed onto the market, caterpillar based production would struggle to deliver high OB yields and in vitro production would likely have a significant advantage (Ravensberg, 2011b; Van Beek and Davis, 2016).

13.3 In vitro production—current status

13.3.1 Introduction

When reference is made to in vitro production of baculovirus biopesticides most reports refer to the production of baculoviruses using suspension culture of insect cells using a fed-batch process in stirred-tank reactors, (typically at a scale of 1000−10,000 L working volumes). The reasons for this are obvious when one looks at the history of Industrial Scale Suspension Culture of Living Cells as outlined by Meyer and Schmidhalter, 2014. These authors point out that suspension cultures in sterile bioreactors have become the standard manufacturing process for producing living cells or products made from living cells and will remain so for quite some time to come. In their chapter on the history and economic relevance of industrial scale suspension culture of living cells, they point out that modern large scale bioreactor processes only started to emerge in the late 19th century but progressed rapidly throughout the 20th century and particularly during the early part of the 21st century with the rise in demand for monoclonal antibodies, other therapeutic proteins and vaccines.

While the first half of the 20th century was dominated by the use of bacteria/yeast and other fungi to produce ethanol, organic acids, solvents and vitamins, this early work improved the design and supply of large-scale sterile bioreactors. The rise in the mass supply of vitamins, steroids and in particular antibiotics from 1930 to 1955 saw a rapid expansion in the extent and scale of sterile bioreactor processes which was further enhanced by the supply of enzymes and amino acids during the 1960s and early 1970s. These products developed the hardware side of large-scale bioreactors to an extensive and sophisticated level (Meyer and Schmidhalter, 2014).

The late 1970s/early 1980s saw the development of recombinant products, initially produced in bacteria, human insulin in 1982, and later the first recombinant protein produced by mammalian cell culture, tissue plasminogen activator in 1986, (Meyer and Schmidhalter, 2014; Alldread et al., 2014). The market for biotherapeutics produced mainly in Chinese hamster ovary cells (CHO cells) grew rapidly from around US$4.4 billion in 1990 to about US$275 billion now, contributing more than 25% of the total pharmaceutical market (Rathore et al., 2021). This market-driven activity in animal cell technology (mostly CHO cell, Monoclonal antibody production-based activity), over a period of 30 years has resulted in a 100-fold reduction in the cost of producing a gram of recombinant protein via animal cell manufacturing processes (Alldread et al., 2014).

Insect cell technology (ICT) (Fig. 13.3) has benefited from this CHO cell technology since the generation of the first recombinant baculovirus in the late 1980s and the Insect Cell/Baculovirus Expression Vector System (IC/BEVS) has gained acceptance as a universal manufacturing platform with the approval of various veterinary and human vaccines (Roldão et al., 2014; Wadman, 2020). Indeed, it has been speculated that large scale suspension culture facilities established for the production of biotherapeutics that may become obsolete due to yield improvements could be deployed for manufacturing of IC/BEVS derived products (Roldão et al., 2014). The availability of such facilities and personnel with skills in large scale animal cell technology may also aid the establishment of an in vitro baculovirus biopesticide production facility assuming the cell line, virus isolate, and media/feed issues discussed below can be addressed.

Given that an OB contains around 0.55 pg of protein per OB (Huynh et al., 2015b), the production of 1.2 g/L of OB protein will result in approximately 2×10^{12} OB/L which is close to a commercially viable yield for the manufacture of baculovirus biospesticides (see Table 13.1). Routine CHO cell technology uses cells that are very similar to insect cells in size and growth rate, plus this technology uses serum-free media and feeds, fed-batch processes, and stirred-tank reactors very similar to the processes which are used for ICT (Alldread et al., 2014; Roldão et al., 2014).

CHO cell technology now routinely produces foreign proteins at 1−5 g/L at a cost of $10/g (Alldread et al., 2014). This animal cell technology is not just present in Europe and the US—but also in India and China and contract animal cell manufacturing is extensive in many countries. A lot of the cost of a pharmaceutical is in the sterile harvest, purification, and sterile formulation of the final product. These downstream costs are low for baculovirus biopesticides as purification is not required (see Fig. 13.4), so $10/g would be on the high side as a cost of manufacturing for baculovirus biopesticides.

This discussion highlighting the maturity of suspension culture technology globally including the suspension culture of animal cell processes is provided to demonstrate that the hardware and skilled personnel are now relatively abundant compared to 20 years ago—so the large-scale capacity for in vitro baculovirus biopesticides to be commercially viable is not a limitation as mentioned above. As discussed below, optimizing the components of the in vitro process at a small scale still presents many challenges but the scale-up technology is in place and potentially available at an appropriate price.

13.3.2 Cell lines available

The three key components of ICT are the insect cell line, the baculovirus vector, and the growth medium. Many insect cell lines have been developed for studies of insect cell physiology, developmental biology, and microbial pathology, and many have been assessed for their ability to produce various baculoviruses, particularly recombinant baculoviruses producing various foreign proteins, vaccines and gene therapy products (Goodman et al., 2001; Mena and Kamen, 2011; van Oers et al., 2015; Chambers et al., 2018; Martínez-Solís et al., 2019). For a cell line to be seriously considered for in vitro production of a baculovirus pesticide it needs to be shown to be capable of a cell doubling time of around 24 hours and to have the capacity to produce a useful virus at a yield of at least 300 OB/cell in suspension culture in a low-cost serum-free media (Claus et al., 2012). Few cell lines have been demonstrated to have these capabilities.

Due to their demonstrated value for producing recombinant proteins for research purposes and for veterinary and human medical applications, the Sf9 (*S. frugiperda*, Smith), and High-Five (*Trichoplusia ni* Hübner) cell lines have been studied extensively in relation to their ability to be grown in suspension culture using serum-free media (Mena

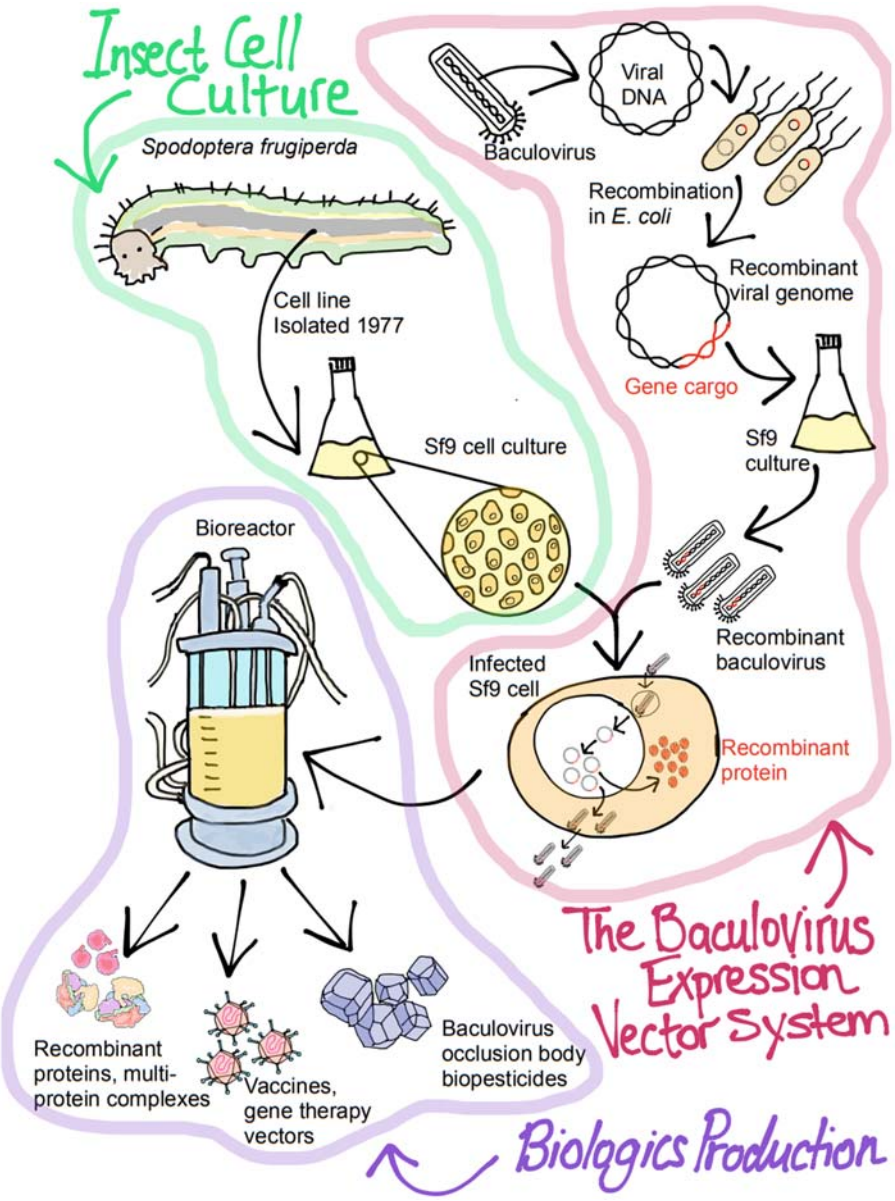

FIGURE 13.3 Applications of ICT and the baculovirus expression vector system. *ICT,* Insect cell technology. *Adapted from a poster presented at the 2019 meeting of Synthetic Biology Australasia, entitled "Harnessing CRISPR/Cas9 to engineer an insect cell factory for enhanced virus production." Figure created by Henry De Malmanche.*

and Kamen, 2011, Huynh et al., 2013, 2015a). In addition, the *H. zea* (HzAM1) cell line produced by Arthur McIntosh (McIntosh and Ignoffo, 1981) has received a lot of attention for its ability to produce HearNPV in vitro (Lua et al., 2002; Nguyen et al., 2011; Pedrini et al., 2011, Huynh et al., 2015b). The Sf9 cell line shows potential for the production of an SfMNPV virus (Almeida et al., 2010; Sihler et al., 2018) and an *A. gemmatalis* cell line has been established that shows potential to produce an AgMNPV product (Micheloud et al., 2009; Micheloud et al., 2011, Claus et al., 2012). No cell lines with strong potential to produce either CpGV or PlxyGV (*Plutella xylostella* (L.) GV), have been reported to date to our knowledge although the High-Five/ *T. ni* cell line may have the potential to produce a PxMNPV, *P. xylostella* MNPV, (Kariuki et al., 2000).

It is worth noting that the Sf9 cell line that has been most widely used has been shown to be persistently infected with an RNA virus known as Sf-Rhabdovirus (Ma et al., 2014), and while this does not appear to affect its ability to produce high virus yields it would seem sensible to develop any future production of an SfMNPV product using an Sf9 cell line that has been rendered Rhabdovirus free (Maghodia et al., 2016). In addition, a clone of the widely used Sf9

TABLE 13.1 Current proposed scale-up process (HearNPV).[a]

Passage no.	Scale (process type)	Virus form added to initiate infection	No. virus particles/ cell added	ICD 10^9 cells/ L	PCD 10^9 cells/L	Product of interest	Yield/ Cell	Yield/L
1	20 L (Batch)	ODV[b]	~400[b]	1	1.0–2.0	BV	50–100	10^{11} PFU/L
2	400 L (Batch)	BV	5.0	1	1.0–1.5	BV	70–100	10^{11} PFU/L
3	10,000 L (Fedbatch)	BV	0.5	4	7.0–9.0	OB	250–300	~ 2×10^{12} OB/L

Note: A single 10,000 L run would produce ~2×10^{16} OB, which is sufficient virus to treat 20–40,000 Ha (assuming an application rate of 0.5–1.0 $\times 10^{12}$ OB/Ha), A single 10,000 L run would produce an equivalent number of OB's that would be produced by 2–20 million infected caterpillars, The scale-up factor in terms of OB produced versus OB required to initiate the process is 100,000X, Each run would take 2–3 weeks to complete.
[a]*Refer to Fig. 13.3 for a schematic of the proposed process.*
[b]*Infection initiated using 10 OB/cell (~400 ODV/cell, assuming ~40 ODV/OB). Initial studies in the Reid lab using PCR to quantify ODV, suggest HearNPV contain 20–40 ODV/OB, so the envisaged P1 infection would require* 2×10^{11} *caterpillar derived OB (assuming* 10^9–10^{10} *OB/caterpillar, indicates that 20–200 infected caterpillars would be required). Acronyms: ODV—occlusion-derived virus, OB—occlusion body, BV—budded virus, ICD—infection cell density, PCD—peak cell density, PFU—plaque-forming unit.*

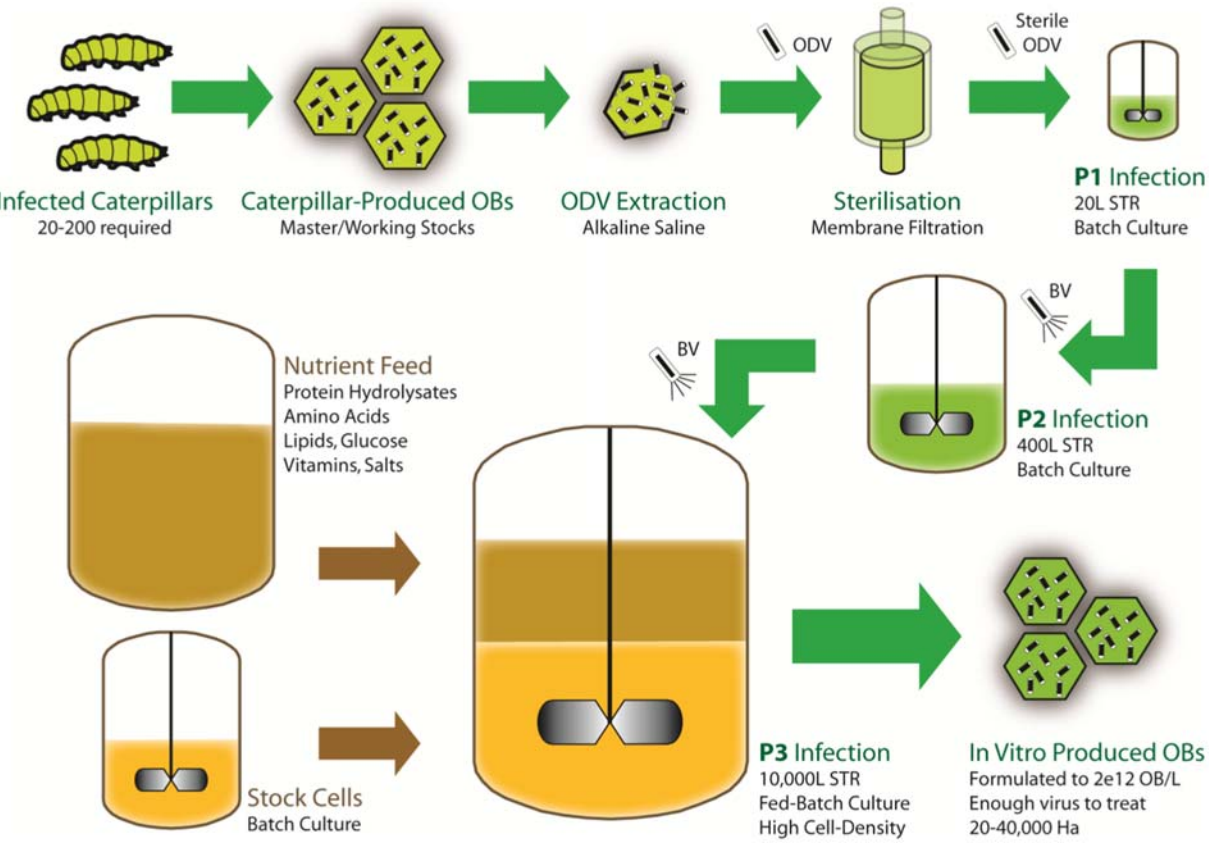

FIGURE 13.4 Current proposed scale-up process (HearNPV)—Schematic diagram (see Table 13.2 for process details). Demonstrates the use of caterpillar-produced HearNPV occlusion bodies (OB) as a source of occlusion-derived virus (ODV), to generate the budded virus (BV) stocks at Passage 1 (P1) and Passage 2 (P2), for infection of HzAM1 cells in the production-scale stirred-tank reactor (STR) at Passage 3 (P3). A high-density fed-batch process is used to deliver high yields of the in vitro produced HearNPV OBs for use as a biopesticide.

cell line and some genetically engineered Sf9 cell lines are available that are reported to improve the productivity of the common "wild type" Sf9 cell line and these deserve to be assessed for the production of SfMNPV OB (Roldão et al., 2014; Smith et al., 2000; Martínez-Solís et al., 2019).

13.3.3 Virus isolates available

For in vitro production of baculoviruses as for in vivo production, the best source of viruses are natural field isolates that show good kill potential for the pest of interest (field isolates from natural epizootics). As viruses passaged in cell culture are subject to genome instability (see Section 13.4.4), in practice the best approach to preserve good field performance for an in vitro produced virus is to minimize the period the virus spends in culture (Reid and Lua, 2005). The approach taken by Reid and Lua, 2005 was to generate master and working stocks of the virus using caterpillar colonies infected by a natural field isolate and then develop a production process that involves infecting cells using ODVs from the OB working stocks produced and allowing the virus to be in culture for only three passages (see Fig. 13.4 and Table 13.1).

For the HearNPV product developed by the Reid group various virus isolates supplied by CSIRO, Division of Entomology, Canberra, Australia were assessed for production in 50 mL shaker cultures using an *H. zea* cell line grown in serum-free media over five passages. Eleven isolates were tested and three showed over 200 OB/cell at passage five with good bioactivity. The best isolate from these three was chosen for further work but all three were likely to be equally suitable for production. Virus isolates that had been purified by many passages within caterpillar colonies or plaque purified via cell culture showed very poor yields at passage five in culture.

The use of SfMNPV ODVs to infect Sf9 cells in culture shows promise for in vitro production of this virus (Almeida et al., 2010), but hemolymph as a source of BV has also shown potential for the in vitro production of AgMNPV (Micheloud et al., 2009; Micheloud et al., 2011; Claus et al., 2012). Given the difficulty of isolating hemolymph and the inherent instability of BV (Jorio et al., 2006), more effort is justified in optimizing the extraction of ODV from OB for infecting cells in culture. The long-term stability of OBs suggests storing valuable master stocks of virus in this form is a much better option than storing BV, and the potential for improving ODV infections of cells in culture is reasonable given the work of Lynn (2003).

Approaches to stabilizing viruses in culture are discussed in Section 13.5.4.4 but if recombinant viruses are to be avoided our recommendation for obtaining virus isolates suitable for in vitro production would be to acquire as many natural isolates as possible against a given pest, screen them using shaker cultures with an appropriate cell line for five passages in culture and store OB from those isolates that continue to produce over 200 OB/cell at passage five.

13.3.4 Low-cost media

The development of insect cell media was originally informed by the chemical composition of insect hemolymph, which was used to design a chemically defined basal medium containing inorganic salts, vitamins, amino acids, organic acids and sugars (Wyatt, 1956). However, the basal medium can only support insect cell growth if it is supplemented with heat-treated hemolymph or vertebrate serum (e.g., fetal bovine serum). Such complex undefined supplements are thought to supply essential growth-promoting components; including fatty acids, sterols and hormones (Schlaeger, 1996). Serum supplementation also allowed the cultivation of insect cells in suspension cultures, including scaling up the cultures in stirred-tank and gas-sparged bioreactors, as serum components can protect cells from shear damage due to mixing and bubble rupture (Goodwin, 1991).

The serum is certainly a more suitable medium supplement than hemolymph, owing to its wide availability. However, the serum is undesirable for large-scale production processes, due to its high cost, lot-to-lot variability, potential harboring of adventitious organisms, and protein content (which interferes with downstream processing). Hence, the next breakthrough in insect cell media was the development of serum-free and protein-free media (abbreviated as SFM), whereby the growth factors, lipids and shear protectants in serum are supplied alternatively by protein hydrolysates and a manufactured lipid emulsion (containing the shear protectant Pluronic F-86) (Inlow et al., 1989; Schlaeger, 1996). This configuration forms the basis of the well-established IPL41 SFM for lepidopteran insect cell lines (Inlow et al., 1989), and its improved commercial derivatives such as EX-CELL 401 (Sigma-Aldrich) and Sf-900™ II (Life Technologies) SFM (Schlaeger, 1996). Further optimization has led to next-generation formulations with reduced hydrolysate content such as Sf-900™ III SFM (Life Technologies).

Conventional lepidopteran SFM is expensive due to the high number of purified amino acids at substantial concentrations in their basal formulations. For example, IPL41 medium contains 21 added amino acids (Schlaeger, 1996),

which account for more than 80% of the total ingredients cost of the complete medium (in-house estimation, based on a uniform assumption of the cheapest nonbulk retail prices for preferably cell culture grade ingredients from Sigma-Aldrich, September 2012). Low-cost serum-free media (LC-SFM) will be required for the development of a low-cost ICT manufacturing platform, suitable for the production of biopesticides, animal vaccines or biosimilars.

LC-SFM can be formulated by replacing most of the added amino acids content in the basal medium with protein hydrolysates, which are generally inexpensive, and are rich sources of free amino acids and oligopeptides, as well as lipids, carbohydrates, vitamins, trace elements, and undefined growth factor analogs (Siemensma et al., 2010). Protein hydrolysates (or peptones) are generally enzymatic digests of crude animal or plant proteins. For convenience, yeast extract is considered a protein hydrolysate in this article, although it is technically an autolysate of yeast cells. Protein hydrolysates from different animal/plant/microbial origins are likely to have divergent amino acid profiles; hence it is prudent to combine various hydrolysates in a low-cost formulation to ensure that there are no amino acid deficiencies. At least five LC-SFM have been described in the literature for lepidopteran insect cell cultures, (Donaldson and Shuler, 1998, Ikonomou et al.; 2001, Schlaeger; 1996, Micheloud et al.; 2009) In addition, the Reid group (at The University of Queensland, UQ) separately developed the low-cost VPM3 SFM in collaboration with Stefan Weiss (Weiss et al., 1992), for HearNPV baculovirus biopesticides production (Reid and Lua, 2005; Huynh et al., 2012). VPM3 contains five added amino acids (asparagine, cystine, glutamic acid, lysine, methionine) and five hydrolysates (3 g/L yeast extract, 2.5 g/L Primatone RL, and 0.5 g/L each of soy hydrolysate, casein hydrolysate and lactalbumin hydrolysate) (Reid and Lua, 2005). VPM3 supported cell densities of around 5×10^9 cells/L (HzAM1) cells, which are around double the volume of Sf9 cells (Marteijn et al., 2003).

When compared to the existing LC-SFM, VPM3 has the disadvantage of containing a higher number of hydrolysates. Each hydrolysate introduces lot-to-lot variability to the production process; hence the quality control monitoring tasks are amplified with each additional hydrolysate. One-hydrolysate VPM3 variants were shown to be significantly worse than their two-hydrolysate counterparts, suggesting that the latter is the lower limit for low-cost formulations with a reduced number of added amino acids (Huynh et al., 2012). When a full complement of added amino acids are present (e.g., in IPL41 basal medium), a single added hydrolysate such as yeast extract is sufficient to derive an efficacious SFM (Maiorella et al., 1988; Inlow et al., 1989).

For a more detailed discussion of LC-SFM for insect cell culture, see Chan and Reid (2016).

13.3.5 Current status of bioreactor-based production—HearNPV as a case study

13.3.5.1 Overview of bioreactor-based processes

Insect cells can be propagated using bioreactor technologies that are optimized for animal cell cultures. Most of these technologies were developed first for mammalian cell cultures, which were in turn adapted from those used for microbial cultures (Agathos, 1991, Meyer and Schmidhalter, 2014). Suitable bioreactor types for suspension-adapted animal cells include the stirred-tank reactor (STR), airlift reactor (ALR) and wave/rocking-motion reactor (Warnock and Al-Rubeai, 2006). The stainless steel STR is by far the most prominent bioreactor type for animal cell-based biologics manufacturing (Warnock and Al-Rubeai, 2006), with the largest reactors having a working volume (V_W) of 20,000 L (De Jesus and Wurm, 2011). Plastic bag-based single-use bioreactors (SUBs) are becoming increasingly popular, but they are currently limited in scale, with the largest systems at 300−500 L V_W for rocking-motion reactors (BIOSTAT RM, Sartorius-Stedim; WAVE Bioreactor, GE Healthcare). Insect cell cultures have been reported at scales of at least 2,000 L in STRs (Cox, 2004, Roldao et al., 2014).

Apart from bioreactor selection, another important consideration for ICT is the culture mode, which includes batch, fed-batch and perfusion. Batch culture is the simplest mode to implement and involves adding fixed volumes of medium and cell inoculum together in the bioreactor, to achieve the desired seeding cell density (SCD). No further nutrient supplementation is carried out apart from oxygen; hence the batch culture volume would be constant if not for periodical sampling. Fed-batch culture is initiated as a batch culture, but when it has reached an appropriate cell density [feeding cell density (FCD)], one or more nutrient supplements (feeds) are added either pulse-wise or continuously, leading to an increase in the culture volume (Elias et al., 2000; Yang et al., 2007). The feeds are usually in the form of nutrient concentrates, which allow higher peak cell densities (PCDs) to be achieved when compared to batch cultures (10^{10} vs 10^9 cells/L orders of magnitude) (Elias et al., 2000). Perfusion culture is also initiated as a batch culture, but the subsequent nutrient supplementation is in the continuous mode, with an inlet (medium) and outlet (harvest) streams at equal flow rates, leading to a constant culture volume (Crowley et al., 2012). Crucially, perfusion culture involves a cell retention device at the outlet stream, which allows the cell density to be increased dramatically (Crowley et al.,

2012). In general, fed-batch culture is preferred for yield maximization on account of its relative simplicity, but challenges may be encountered in terms of formulating feed concentrates and the prospect of toxic metabolites buildup. On the other hand, perfusion culture provides an optimal physio-chemical environment. Through continuous removal of spent medium and toxic metabolites, perfusion culture can achieve much higher PCDs at the 10^{11} cells/L range (Adams et al., 2011; Crowley et al., 2012), but the trade-off is higher process complexity.

Bioreactor-based ICT processes for recombinant proteins have been developed in batch (5×10^9 cells/L, uninfected) (Maiorella et al., 1988), fed-batch (up to 5.2×10^{10} cells/L, uninfected) (Elias et al., 2000; Bedard et al., 1997) and perfusion (up to 5.5×10^{10} cells/L, uninfected) (Caron et al., 1994; Deutschmann and Jager, 1994) modes, since the late 1980s. ICT processes for baculovirus biopesticides have been developed mainly in shaker/spinner flask batch cultures at relatively low cell densities and modest OB yields (Table 13.2). However, some of these batch processes have been scaled up in bench-scale bioreactors, for example, wt-AgMNPV (2×10^{11} OB/L) (Micheloud et al., 2011) and wt-CfMNPV (6×10^9 OB/L) (Meghrous et al., 2000). In one particular case, wt-AcMNPV has been scaled up in a bench-scale perfusion bioreactor (1.6×10^{10} OB/L) (Kloppinger et al., 1990).

13.3.5.2 Maximum fed-batch yields reported

Batch cultures are productive only up to an optimum PCD (postinfection), which is medium-specific. For example, Sf9 cells grown in Sf-900 II SFM and infected with recombinant AcMNPV (expressing nonsecreted b-Galactosidase) can only support optimal specific yields (SY, units/cell) up to a PCD of around 4×10^9 cells/L, above which a dramatic decline in productivity is observed, even though the uninfected PCD for this medium is two to threefold higher (Wong et al., 1996; Huynh et al., 2013, 2015a). In other words, the volumetric yield (VY, units/mL) initially increases linearly with PCD, then reaches a maximum, and finally declines rapidly at higher PCDs (Wong et al., 1996; Radford et al., 1997). This phenomenon is largely correlated to the PCD, and appears to be independent of the multiplicity of infection (MOI) and the infection cell density (ICD), which informs the "cell yield concept" (Wong et al., 1996).

The decline in productivity of batch cultures can be reversed by adding more nutrients. Hence, the fed-batch mode is a means of maximizing the VY of a production system by elevating the PCD at which the optimal SY is maintained. Over the past two decades, much progress has been made to improve the yield of ICT-expressed recombinant proteins using fed-batch cultures. One of the earliest fed-batch studies involved a single pulse-addition of a multicomponent serum-free feed to Sf9 cells grown in Sf-900 II SFM (Bedard et al., 1994), which could extend the optimal infected PCD to around 7×10^9 cells/L, for b-Gal production in shaker flasks. Similar feeds were subsequently tested which extended the optimal infected PCD to around $7-14 \times 10^9$ cells/L, (Bedard et al., 1997; Chan et al., 1998; Chan et al., 2002). The fed-batch bioreactor processes developed, when tested at higher PCDs than optimal, at 14×10^9 and 17×10^9 cells/L, resulted in cell-specific yields that were around 20% and 50% lower respectively (Elias et al., 2000), when compared to optimal cell-specific yields. Other fed-batch studies also tested the efficacy of the reported feeds, showing that optimal yields can be maintained at PCDs of $9-10 \times 10^9$ cells/L in bioreactors, for ICT-expressed influenza vaccine (Meghrous et al., 2009) and rAAV production (Mena et al., 2010).

In the Reid group, over a decade has been devoted to the development of a scalable fed-batch bioreactor process for HearNPV biopesticides produced in HzAM1 cells, in collaboration with Agrichem (a leading Australian liquid fertilizer company). This work followed extensive studies in elucidating and modeling ICT processes for the Sf9/rAcMNPV system using batch/fed-batch modes (Power et al., 1994; Wong et al., 1996; Radford et al., 1997; Chan et al., 1998; Jang et al., 2000; Rosinski et al., 2002; Haas and Nielsen, 2005), and in characterizing the HzAM1/HearNPV system in batch

TABLE 13.2 Baculovirus biopesticides production in insect cell suspension batch cultures using shaker or spinner flasks, as reported in the literature.

Virus	Cell line	Flask	PCD cells/L	VY OB/L	References
AcMNPV	Tn5B14	Spinner	1×10^9	2×10^{11}	McKenna et al. (1997)
AgMNPV	UFLAG286	Spinner	1×10^9	6×10^{10}	Gioria et al. (2006)
HaSNPV	HzAM1	Shaker	2.6×10^9	7×10^{10}	Chakraborty et al. (1996)
SfMNPV	Sf9	Shaker	1×10^9	5×10^{11}	Almeida et al. (2010)

Note: For each case, the peak cell density (PCD) postinfection, and the volumetric yield (VY) of virus occlusion bodies (OBs), is indicated.

mode (Chakraborty et al., 1999; Chakraborty and Reid, 1999; Lua and Reid, 2000; Lua et al., 2002; Pedrini et al., 2006; Pedrini et al., 2011). A major difference between the fed-batch processes for HearNPV and those in the past is that both the medium and feed were low-cost formulations, due to the narrow economic margins required for a biopesticide product. As described in Section 13.3.3, our in-house LC-SFM (VPM3) relies heavily on protein hydrolysates to minimize the requirement for expensive pure amino acids, and this reliance was carried forward for the low-cost serum-free feed designs. The HearNPV fed-batch process was optimized by employing a heuristics-based optimization strategy informed by the "cell yield concept" (Wong et al., 1996), design of experiments (DoE) techniques (e.g., fractional factorial and response surface designs), and the Power-Nielsen model of baculovirus infection (Power et al., 1994). The feeds developed by the Reid group supported HearNPV OB yields of 2×10^{12} OB/L, which to the best of our knowledge, is the highest reported for baculovirus OBs generated via an in vitro production system.

For a more detailed discussion of serum-free feeds for insect cell culture, see Chan and Reid (2016).

13.3.5.3 Economic feasibility

Significant examples of the deployment of in vivo-produced baculovirus biopesticides were mentioned above (Section 13.2), hence there are sizable existing markets for baculovirus products, which may expand with the advent of more scalable and reliable in vitro production (Ravensberg, 2011b; Grzywacz and Moore, 2017).

The economic feasibility of an ICT-based in vitro production process for baculovirus biopesticides was analyzed previously, using an established fermentation production cost model (Rhodes, 1996). In this case, an economic process is obtained at a final production bioreactor scale of 225,000 L, if 4 bioreactors are employed and 162 batch cultures are run annually. Assuming a volumetric OB yield of 4×10^{11} OB/L per run, this process was estimated to have a manufacturing cost of USD 2/L culture, an operating margin (return on sales) of 29%, and enough product to treat 11.7×10^6 Ha/year. However, such a process is unlikely to be implemented as animal cell culture STRs are currently limited to a maximum scale of 20,000 L working volume, as discussed previously (Warnock and Al-Rubeai, 2006; De Jesus and Wurm, 2011; Roldao et al., 2014; Alldread et al., 2014). Another baculovirus biopesticide production scheme (for AfMNPV) was postulated to be economically viable at a production scale of 20,000−50,000 L, with a manufacturing cost of USD 2−3/L product, but the production targets were not specified (Jem et al., 1997).

The Reid group also developed a comprehensive business plan (unpublished) for the production of HearNPV in HzAM1 cell cultures, using a high cell density fed-batch process (as mentioned in Section 13.3.5.2). At currently achieved fed-batch yields (2×10^{12} OB/L); a production facility based on a final bioreactor scale of 10,000 L working volume and 23 production runs/Yr appears to be economically feasible. In this case, around 200,000 L/year of the formulated product (2×10^{12} OB/L) will be manufactured, which is estimated to have a manufacturing cost of AUD 19/L and a sale price of AUD 50/L, resulting in an operating margin of 39%. Formulated HearNPV OBs (from 100 L fed-batch bioreactor runs) were shown to be as efficacious as in vivo-produced controls in field trials and were registered as "Heliocide" with the Australian Pesticides and Veterinary Medicines Authority (APVMA) in 2008.

There is of course much scope to improve the economics of the current HearNPV process. For example, the capital and operating costs may be substantially reduced by locating the production facility in established CHO cell bioreactor facilities that have become redundant due to yield increases for biotherapeutics as outlined in Section 13.3.1. Further cost efficiencies will be obtained by increasing the existing volumetric OB yield. This may be achieved by increasing the PCD of fed-batch infections, which requires the development of improved nutrient feeds. Since current feeds are already at the solubility threshold (in relation to the hydrolysates content), further feed improvements will need to be more targeted, by incorporating elements of chemically defined formulations, as elaborated in Sections 13.4.1 and 13.5.1 below.

13.4 Limitations to bioreactor production of baculovirus-based pesticides

13.4.1 Lack of a chemically defined media

The reliance of low-cost media on protein hydrolysates to supply growth factors and a majority of amino acids has a major disadvantage, that being the lot-to-lot variability of such undefined components (Kasprow et al., 1998; Zhang et al., 2003). Since the source material and the manufacturing process of a hydrolysate may be variable, the chemical composition of the end product may also be variable, which leads to variability in the medium and ultimately the cell culture process (Zhang et al., 2003; Pasupuleti and Braun, 2010). Ultrafiltration (e.g., 10,000 MW cut-off) of the hydrolysate may help improve process consistency (by removing endotoxins) (Pasupuleti and Braun, 2010), but the underlining variability in chemical composition is still unresolved.

Apart from this concern, hydrolysates are an untargeted form of nutrient supplementation, since many other components, some of which may be undesirable, are added together with the desirable components such as amino acids, oligopeptides, and small-molecule growth factors. For example, hydrolysates can have high inorganic salts content (Pasupuleti and Braun, 2010), and some undefined components may impart cellular toxicity (Lu et al., 2007). Furthermore, hydrolysates can have widely divergent aqueous solubility, whether they are from the same or a different source material. For example, the yeast extract HyYest 444 (Sheffield Biosciences) may exhibit solubility's of between 50 and 300 g/L depending on the lot number (as tested by the Reid group). Hence, constraints are likely to be encountered as the concentration of hydrolysate(s) is increased, in particular when formulating concentrated low-cost nutrient feeds for fed-batch cultures, in terms of osmolality, toxicity and solubility limits.

The problem of hydrolysate variability may be addressed by first developing a chemically defined medium (CDM), from which knowledge is gained in what chemical(s) can be used to supplement a "bad" batch of hydrolysate so that it matches the performance of a "good" batch. In this way, hydrolysates can continue to be used in media and feeds to preserve the low-cost nature of the process, which is necessary for baculovirus biopesticide production. Currently, commercial CDM formulations are well established for mammalian cell cultures, which reflects the industrial importance of mammalian cell-expressed biologics (Mena and Kamen, 2011).

Recently Thermo Fisher Scientific developed a chemically defined media and enhancer (ExpiSf CD Medium and ExpiSf Enhancer), for use with Sf9 cells and the BEVS system. While this commercial product is competitive with the best serum-free media and feeds available, (assessed in the Reid lab, unpublished), it is too expensive to use to produce biopesticides and its formulation remains confidential. However, this development clearly shows that defined substitutes for the yeast extract, an essential ingredient in serum-free insect cell media and feeds, have been identified. This should encourage future efforts to develop low-cost feeds which are limited by the amount of concentrated yeast that can be added in fed-batch cultures due to excessive toxins that accumulate in the culture as discussed above.

Defined components of media are expensive to identify but if used in trace amounts are not necessarily expensive to use. Animal cell CDM formulations are expensive as they are sold at prices that the pharmaceutical and research market will bear but which do not necessarily reflect the cost of their manufacture.

Strategies for developing an insect CDM are described in Section 13.5.1.

13.4.2 Low budded virus titers

The baculovirus form required for efficient cell culture infections is the BV. The BVs to be used as infectious seeds for bioreactors can be produced in larvae and extracted from hemolymph, but this is not an ideal process for large scale production. Therefore, an initial round of OB-derived ODV can be used to generate BVs in cell culture (Lynn, 1994, 2003; Reid and Lua, 2005; Almeida et al., 2010), which can be followed by an extra round of amplification in small size bioreactors (see Fig. 13.4).

A major limitation to reproducible production is the variability in the initial infection resulting from the poor recovery of infectious ODV from the ODV extraction/filtration process used. Optimizing this step has been neglected due to poor options to assay ODV numbers and quality during the extraction/filtration process. However, PCR has emerged as a reliable and relatively simple way to monitor ODV numbers (Pedrini et al., 2011; George et al., 2012). Work to date by the Reid group indicates that significant numbers of ODV are lost during the filtration process and Electron Microscopy studies suggest severe clumping of the ODV occurs. Optimization of this process needs to be addressed and is likely to improve the consistency of the genetic material supplied into the production process at Passage 1, potentially leading to more reproducible BV and OB yields from the subsequent passages.

For large-scale production in bioreactors the infectious titer of the BV seed stock is important. The number of infectious virions per volume is normally determined in a plaque assay (plaque-forming units) or an end point dilution assay [tissue culture infective dose 50 ($TCID_{50}$)], and may differ considerably from the number of nucleocapsids measured as genome copy numbers using Q-PCR. For AcMNPV high BV titers are obtained in Sf9 (Vaughn et al., 1977; Matindoost et al., 2014) and *T. ni* High-Five (Wickham et al., 1992) cell cultures (routinely up to $1-10\times10^{11}$ PFU/L for Sf9 cells). For many viruses, though, the BV titers obtained after even a single passage in cell culture are often lower than in hemolymph. For HearNPV for instance infectious BV titers are in general 10 times higher in hemolymph than in culture medium, where titers of 1×10^{10} to 5×10^{10} $TCID_{50}$/L are obtained with HzAM1 cells. In Hz2e cells titers up to 1×10^{11} $TCID_{50}$/L are seen (Fei Deng, personal communication). Another example is *Trichoplusia ni* NPV, for which virus titers are much lower than for HearNPV and drop even further after one passage in *T. ni* High-Five cells (e.g., $\sim1.75\times10^{9}$ $TCID_{50}$/L compared to $\sim$ 5.27×10^{8} $TCID_{50}$/L, Martin Erlandson personal communication).

In addition, the same batch of the virus may give varying levels of infection in various cell lines. The same batch of AcMNPV BV virus for instance gives higher titers when tested on *T. ni* High-Five than on Sf9 cells (nonpublished data). Variation in BV titers may also exist between virus strains as shown for *Mamestra configurata* (Maco) NPV strains in the *Mamestra brassicae* (Linnaeus) cells IZD-MD-0503 (American Tissue Culture Collection). These MacoNPV plaque isolates have variable efficiency of replication in the *M. brassicae* cells, but the best isolates produce titers of a maximum of 4.5×10^9 $TCID_{50}$/L and some were as low as 1.5×10^8 $TCID_{50}$/L (Martin Erlandson, personal communication).

In conclusion, we can say that the infectious BV titer produced in cell culture varies strongly per virus (species/isolate) and may depend strongly on the cell line used for amplification and for testing. This means that to obtain optimal infectious BV titers, careful selection of cell lines and procedures may improve performance. However, as baculoviruses in general have a restricted host range, the choice of permissive cell lines is often limited. For GVs, much fewer cell lines have been shown to be permissive (6 lines for 5 viruses, compared to over 150 cell lines for more than 30 NPVs, Dwight Lynn personal communication). For an overview of different insect cell lines see (van Oers and Lynn, 2010).

13.4.3 Occlusion-derived viruses produced in cell culture may have a lower speed of kill

ODVs produced in larvae may show an increased speed of kill compared to ODVs produced in cell culture as was shown for AcMNPV (Bonning et al., 1995). The lethal viral dose needed for 50% mortality, however, does not differ between ODVs produced in larvae and cell culture. The reason for the difference in lethal time is not clear, but an important contribution may be the status of one of the PIF proteins. AcMNPV ODVs derived from infected larvae contain a proteolytically processed P74 (PIF0) protein, consisting of two fragments of about equal size (Peng et al., 2011). This proteolytic cleavage of P74 occurs as soon as OBs derived from infected larvae are dissolved under alkaline conditions. On the contrary, ODVs derived from cultured cell lines did not show this cleavage of P74. Identification of the responsible alkaline protease could provide a method to genetically modify cultured cells so that these cells can also produce the protease necessary to cleave P74 upon ODV release from OBs. In this way, cultured cells may be programmed to produce viral OBs with a similar speed of kill as OBs produced in larvae.

The cleavage of P74 into two equally sized parts as described above precedes a second cleavage, mediated by midgut trypsin. This second cleavage occurs more toward the N-terminus of the P74 protein in both cell and larvae-derived ODVs. This second cleavage is needed to fully activate the P74 protein (Slack et al., 2008). The fact that P74 is an ODV surface protein that is cleaved in the midgut prior to virus entry, suggests that P74 is a crucial component of a viral fusion complex.

13.4.4 Viral genome instability during in vitro passaging

13.4.4.1 Natural virus populations

Baculovirus species are often found in nature as genotypic mixtures. Such mixed genotypes are common for instance for *Spodoptera exigua* (Se) MNPV (Munoz et al., 1998), *S. frugiperda* (Sf) MNPV (Simon et al., 2005b; Simon et al., 2006), and *Malacosoma californica pluviale* (Mcpl) NPV infecting the western tent caterpillar (Cooper et al., 2003). For McplNPV a relation between host density and composition of the viral genetic pool has been reported (Cooper et al., 2003). Genetic mixtures may even occur within a single larva as reported for *Panolis flamea* (Pf) NPV, where up to 24 variants were seen within a single insect (Cory et al., 2005).

The individual isolates may vary in speed of kill and/or lethal dose, and sometimes an isolate has completely lost oral infectivity capacity, meaning this isolate was dependent on other genotypes in the population to start a new round of infection (Cory and Myers, 2003; Lopez-Ferber et al., 2003; Kemp et al., 2011). A large screen among virus isolates from heliothine pest insects (*H. armigera, H. virescens and H. zea)* allowed the grouping of isolates based on a selection of sequenced genes and also detected several mixed populations (Rowley et al., 2011). A selection of the isolates was tested against *H. zea* larvae and compared to the commercially available Gemstar biocontrol product. The commercial product killed the larvae faster than most other Hz/HaSNPV and HearMNPV isolates. On the other hand for Gemstar and two HearMNPV isolates more virus was needed to kill 50% of the larvae than for the other Hz/HearSNPV isolates tested (Rowley et al., 2011), demonstrating that speed of kill and lethal dose are two different parameters of virus isolates that are not per se correlated. When developing biocontrol products, one must determine whether the natural mixture is preferable in terms of dose needed and time to kill, or whether one of the isolates performs better. As the composition of mixtures may change during production in bioreactors, methods may be needed to control and determine the composition of mixtures.

13.4.4.2 Selection and generation of mutants in cell culture

When baculoviruses are passaged in cell culture (of course in the form of BVs) certain genotypes are easily selected for and previously nonexisting deletion mutants may be generated. One obvious reason is that many functions can be omitted when the virus does not have to be transmitted to a new host insect. For instance, in cell cultures, there is no need to retain ODV-specific proteins, such as the PIF proteins, the major OB protein or viral proteins that regulate OB yield.

Amplification of SeMNPV in *S. exigua* Se301 or SeIZD2109 cells leads to large deletions in the *Xba*I-A fragment, roughly the region from the open reading frame (ORF) 16–36 (Heldens et al., 1996; IJkel et al., 1999; Pijlman et al., 2003c). Often such mutants have lost the ability to orally infect larvae and this finding actually led to the discovery of oral infectivity factor PIF2 (Se35, homologous to Ac22) (Pijlman et al., 2003a). To avoid the problem of obtaining viruses without in vivo infectivity, an alternating cell culture/larval cloning strategy was developed. This procedure resulted in a mutant (Se-XD1) with a deletion affecting ORFs 15–28. This mutant was able to infect larvae orally and was already present in the original virus isolate (Dai et al., 2000). Mutant Se-XD1 lacked, amongst others, the *egt*, chitinase and cathepsine genes. The *egt* gene encodes ecdysteroid uridine 5′-diphosphate (UDP)-glucosyltransferase, an enzyme that increases OB yields by suppressing the molting of infected larvae (O'Reilly and Miller, 1989; Cory et al., 2004). Chitinase and cathepsin break down the chitin skeleton of diseased insects, and as such, assist in the release of OBs from cadavers (Hawtin et al., 1997). These three enzymes are important for efficient virus transmission in nature, but there is apparently not as strong selection pressure on these genes under laboratory settings.

So, in cell culture, genetic variants may be selected from already existing genotypes, or new genotypes may be generated. An extreme example of the latter is the formation of defective interfering (DI) particles. DIs appear as soon as baculoviruses are passaged outside insect larvae in cultured insect cells (Pijlman et al., 2001) and easily accumulate in bioreactor runs (Kool et al., 1991). DI particles have a reduced size due to large deletions in the viral genome (Kool et al., 1991; Lee and Krell, 1992). DIs show parasitic behavior toward the wild type virus, as DIs depend on functions encoded by full-length genomes in the population to replicate. At the same time, DIs provide strong competition to the replication of full-length genomes, since the smaller DIs complete their replication quicker. When AcMNPV expression vectors are used in bioreactors, DI accumulation leads to a reduction of recombinant protein production and prevents the application of continuous baculovirus-insect cell production systems (van Lier et al., 1990). As the heterologous genes are normally inserted in the polyhedrin locus, one can predict, that when using wild type virus as starting material, the level of polyhedrin production may drop in a similar way, leading to a reduction in OB formation.

For AcMNPV, defective genomes of approximately 50 kbp have been reported that contained re-iterations of baculovirus sequences homologous to 2.2% of the viral genome (Lee and Krell, 1994). As these genomes were replicating, it was concluded they had to contain an origin of replication (ori). As this origin was not similar to the already known origins in the form of homologous regions (hr), which consist of tandem and inverted repeats and are dispersed over the genome, the new sequence was named a *non-hr* ori (Kool et al., 1994). In AcMNPV this region is located within the nonessential *p94* gene. For SeMNPV intracellular DNA circles were reported with iterated *non-hr* sequences in Se301 cells (Pijlman et al., 2002). When SeMNPV and AcMNPV were engineered to remove the *non-hr*, the viruses replicated well and the genomes were stabilized (Pijlman et al., 2002, 2003b). Deleting the *non-hr* element appears to be a good method to stabilize baculovirus genomes in general, but so far has hardly been applied.

From the above it is clear that the simplest method to keep DI accumulation to a minimum is to limit the number of passages in cell culture and to use low multiplicities of infection when preparing BV seed stocks, so that wild type viruses cannot provide helper functions for deletion mutants. The fact that deletion mutants enriched in *non-hr* sequences were effectively removed within two passages when larvae were infected with OBs produced in cell culture, clearly indicates that DIs are something artificial (Zwart et al., 2008a). In vivo passaging may, therefore, be used to clean up virus stocks propagated in cell culture. Using OBs produced in larvae as a seed stock may assist to avoid problems, but this process needs technological development, for example, in terms of ODV extraction, filtration to remove bacteria and fungi and quality control (see Section 13.4.2).

Methods to measure accurately the accumulation of DIs are needed to monitor baculovirus infections in cell culture and to see how quickly DI formation becomes a problem in a particular combination of virus and cell line. A quantitative PCR method was developed for AcMNPV that compares levels of the immediate-early gene 1 (*ie-1*) with that of *non-hr* sequences (ideally the ratio = 1:1) (Zwart et al., 2008b) and this tool can easily be adopted for other viruses. Such methods will also assist to determine whether adaptations in the production process or modifications to the genome have a positive effect by delaying DI accumulation.

13.4.4.3 Instability through transposable elements

Important factors that contribute to the plasticity of baculovirus genomes include homologous recombination between related baculoviruses, and internal recombination events facilitated by the reiteration of origins of replication (*hrs*) and

the presence of baculovirus repeat ORFs (*bro's*), as well as transposon insertions. Transposons are mobile genetic elements that are mediators of genomic plasticity by promoting recombination between genomes and the hypothesis is that they have played an important role in establishing the composition of baculovirus genomes (van Oers and Vlak, 2007). Transposable elements can result in gene insertions, gene interruptions and even inversions of genome fragments. Transposons may also alter the expression of genes flanking the insertion site. The transposons in genetic variants of *C. pomonella* GV (CpGV) are members of the *Tc1-like/mariner* transposon family. They are no longer mobile as they encode defective transposase enzymes (Jehle et al., 1995; Jehle et al., 1998) and comprise insertions of several kbp derived from the hosts *Thaumatotibia (Cryptophlebia) leucotreta* (Meyrick) and *C. pomonella*. The transposon from *C. pomonella* leads to variation in the form of inversions between CpGV strains through homologous recombination between its inverted terminal repeats (Arends and Jehle, 2002).

Insertion of mobile elements in baculovirus genomes also occur during propagation in lepidopteran cell cultures and contributes to genome instability and may lead to reduced OB formation. A hotspot for transposon insertions in AcMNPV is the *fp25K* ORF (Fraser et al., 1985; Beames and Summers, 1988).

Insertions in this locus lead to the "few polyhedra or FP" phenotype, which is often seen when the virus is passaged in cell culture and is characterized by fewer OBs with fewer or no ODVs inside (e.g., Hink, 1976; Knudson and Harrap, 1976; Fraser and Hink, 1982). As the name implies, FP mutants have a reduced yield of OB production and this is accompanied by reduced levels of the FP25K protein. The FP25K protein plays a role in the intracellular transport of ODV envelope proteins (Braunagel et al., 1999; Braunagel et al., 2004) and as such is involved in the biosynthesis of biologically active ODVs. As a consequence, insertions in this locus affect the efficacy of OB preparations.

The most frequent transposon-mediated insertions in baculoviruses are relatively small and are inserted specifically at TTAA target sites, such as the 634 bp insertion found in AcMNPV isolate E amplified in *S. frugiperda* cells (Schetter et al., 1990). The hitchhiker element, derived from T. ni cells, is 579 bp long and inserts at TTA sites (Bauser et al., 1996). Other examples with high specificity for TTAA sites are the tagalong (TFP3) (Wang et al., 1989) and piggyBac (IFP2) elements (Cary et al., 1989) that may be inserted when the virus is amplified in *T. ni* cell lines. These elements normally do not have ORFs and are characterized by small, inverted repeats. These types of insertions play a major role in the appearance of the FP phenotype mentioned above. After insertion of tagalong and piggyBac, revertants have been recorded that result from precise excision (Fraser et al., 1996).

In order to stabilize the *fp25k* locus, recent experiments aimed at altering the TTAA motifs in the *fp25k* gene that are prone to transposon insertion, while leaving the encoded amino acid sequence intact. This indeed stabilized the synthesis of the FP25K protein and appeared to delay the accumulation of the FP phenotype, but the envelopment of nucleocapsids and the occlusion of virus particles were aberrant (Giri et al., 2011). The FP phenotype still appeared, despite continued FP25K protein synthesis, indicating that other events can also lead to an FP phenotype. This is in line with reports of transposon insertions in for instance the AcMNPV *94k* and *da26* genes, that also gave an FP phenotype (Friesen and Nissen, 1990; O'Reilly et al., 1990).

Larger insertions in the FP25k locus may result from the transposition of a *copia-like* retro-transposable element, called TED (gypsy family), which is derived from the *T. ni* genome (Miller and Miller, 1982; Friesen and Nissen, 1990). TED insertions are characterized by long terminal repeats and carry *gag, pol* and *env* genes (Ozers and Friesen, 1996; Hajek and Friesen, 1998). Excision of the TED element from an AcMNPV FP mutant left behind a long terminal repeat (LTR) that showed promoter activity in both directions (Friesen et al., 1986). This is an example of how a transposon insertion and imperfect excision may alter the gene expression of flanking genes. Errantiviruses have probably evolved from a TED retrotransposon that obtained a copy of the baculovirus F gene, thereby converting the retrotransposon into an insect retrovirus (Malik et al., 2000; Pearson and Rohrmann, 2004). Although data on transposons are available only for a limited number of cell types it is likely that similar elements act in other cells/virus systems.

13.4.5 Complications with high-density cell culture

High-density cell culture is attractive in terms of saving space and medium. On the other hand, complications may arise in providing nutrients and oxygen, and at the same time avoiding shear stress. Under starvation conditions, which may occur under high cell density, cell division is impaired and apoptosis is easily induced (Laken and Leonard, 2001). Controlling important metabolic processes is therefore extremely important when growing high-density cultures, for a review see (Ikonomou et al., 2003). Baculovirus infection requires dividing cells and high cell densities give a drop in specific production of recombinant proteins in the baculovirus expression systems (see Section 13.3.5.2). Similar effects are known to occur for OB production (Chakraborty et al., 1996). Metabolic flux analysis, whereby levels of many different metabolic compounds were followed, revealed that the central metabolism of Sf9 cells is progressively inhibited

when cell densities increase (Bernal et al., 2009). High cell density affected the incorporation of amino acid carbon backbones into the citric acid cycle and down-regulated glycolysis. Modulation of the energetic status of the culture by adding pyruvate or alpha-ketoglutarate at the time of infection resulted in 6–sevenfold higher yields of BV at high cell density when compared to control cultures and resulted in higher titers than classical low cell density infections (Carinhas et al., 2010). A recent proteomic study has revealed more cellular components that may be modulated to improve baculovirus infections under high cell density conditions (Carinhas et al., 2011).

13.5 Future research directions for bioreactor production of baculovirus-based pesticides

13.5.1 Chemically defined media for insect cell culture

As described in Section 13.3.4, conventional insect cell media are composed of a chemically defined basal medium, which has to be supplemented with complex undefined additives such as vertebrate sera or protein hydrolysates, and an undefined or defined lipid emulsion for serum-free formulations, to support cell growth and baculovirus production. As Thermo Fisher Scientific has already developed a chemically defined media for Sf9 insect cells (see Section 13.4.1), then it should be feasible for others to achieve this outcome.

Commercial CDM formulations are widely available for mammalian cell cultures (e.g., CD OptiCHO, Life Technologies; PowerCHO, Lonza; IS CHO-CD, Irvine Scientific). Insects have generally similar nutritional requirements to that of vertebrates, and insect and mammalian basal media share many similarities in chemical composition (Schlaeger, 1996). Furthermore, the development of insect cell media was highly influenced by that of mammalian cell media, in terms of the substitution of vertebrate sera with protein hydrolysates, and the use of Pluronic F-68 as a cell protectant to enable cell growth in suspension cultures (Schlaeger, 1996). In addition, studies have shown that insect cells can be adapted to grow in mammalian media, and even co-cultured with mammalian cells; for example *Agallia constricta* (Van Duzee), (Order: Hemiptera), or *T. ni* (Order: Lepidoptera), cells co-cultured with mouse fibroblast cells in TC199-MK serum-containing medium (McIntosh et al., 1973; Epstein and Gilula, 1977). Nevertheless, insect cells do require certain conditions for optimal cell growth and baculovirus infection, that are divergent from that of mammalian cells (Schlaeger, 1996; Drugmand et al., 2012). Firstly, sterols are not synthesized by insect cells, hence they have to be supplemented in the medium (e.g., cholesterol in serum or in a lipid emulsion). Secondly, insect cells prefer lower pH (6.2–6.9 vs 7.0–7.3), lower temperature (22°C–29°C vs 33°C–37°C), and higher osmolality (340–390 vs 290–330 mOsmol/kg) settings, when compared to their mammalian counterparts. Furthermore, insect media are usually formulated with higher levels of free amino acids and organic acids to mimic the composition of hemolymph (Schlaeger, 1996)

For Sf9 insect cell cultures, an efficacious serum-free medium can be prepared by supplementing IPL41 basal medium with chemically defined lipids (Life Technologies) and yeastolate ultrafiltrate (Life Technologies), which can support PCDs of around 14×10^9 cells/L in our hands. However, the PCD declines with decreasing yeastolate content, and no cell growth is recorded after the second passage if yeastolate is omitted (unpublished data). Hence yeastolate is highly critical for insect cell proliferation in IPL41 medium, and the main challenge in developing an insect cell CDM is to replace the complex functionalities of such undefined hydrolysates. One potential solution is to fractionate and determine the active components of yeastolate (Shen et al., 2007) or other hydrolysates, which can then be chemically identified for inclusion in the basal medium. Another approach is to combine aspects of existing fully disclosed mammalian CDM formulations with basal insect media such as IPL41, while taking into account the specific sterol and physico-chemical requirements of insect cells. Examples of mammalian CDM formulations include CDSS (Qi et al., 1996), MET1.5 (Epstein et al., 2009), and CD-CHO (Gorfien et al., 2012). MET1.5 and its affiliated formulations are demonstrated to be efficacious for hybridoma cell culture and monoclonal antibody production (20×10^9 cells/L) but are also claimed to be suitable for the culture of eukaryotic cells in general, including insect cells (Epstein et al., 2009). Suitable chemically defined growth proliferation factors (as hydrolysate substitutes) may include efficient forms of chelated iron (Epstein et al., 2009; Gorfien et al., 2012), synthetic oligopeptides (Franek et al., 2003), trace elements such as sodium selenite (Qi et al., 1996; Popham and Shelby, 2007), and antitoxic factors (Qi et al., 1996).

Additionally, there are a limited number of fully disclosed (uncommercialized) insect cell CDM formulations in the literature (Wilkie et al., 1980; Becker and Landureau, 1981; Mitsuhashi and Goodwin, 1989; Mitsuhashi, 1996). However, these CDM formulations are generally preliminary, complex, not well-validated, and tested using poorly defined stationary cultures.

In summary, it is feasible to formulate an insect cell CDM for industrial applications, given the success of Thermo Fisher Scientific in this regard and many published formulations of mammalian and even some insect CDM media are available to provide ideas of what to test in order to develop additional CDM's for insect cells. While it may be a challenging task to duplicate the Thermo Fisher Scientific success, with appropriate funding it should be feasible. An insect cell CDM may then allow the development of more reproducible hydrolysate-based low-cost media and feeds, which would be beneficial in the development of a commercially viable baculovirus biopesticide process.

13.5.2 Genomics/transcriptomics of insect cell lines

Further improvements in OB yields via in vitro production processes will likely require gene modifications of the host cell lines in order to reduce the cell lines' ability to attenuate viral pathology (Harrison and Hoover, 2012). It is not an option to genetically modify the virus used if the product is to be accepted as a wild type and therefore maintain the ability to avoid severe registration and environmental concerns. Genetically modifying the host cell line to improve yields requires first identifying the best host gene targets to up or downregulate. This in turn requires a detailed assessment of the host cell gene response to a virus infection. Ideally, the genome of the host insect cell lines would be available to allow appropriate studies in this regard to be made.

Li et al. (2019) reported that 155 annotated insect genomes are available based on the National Centre for Biotechnology Information (NCBI) records of which 25 are for Lepidoptera, while Triant et al. (2018) produced a list of 30 Lepidoptera genomes with functional annotations as of Dec 2017. Of the 30 Lepidoptera annotated genomes listed by Triant et al. (2018) 25 of them only became available in 2015. In relation to research aiming to improve in vitro production of baculoviruses it is useful to know that good, annotated genomes are now available for *S. frugiperda* (Xiao et al., 2020), *H. armigera* and *H. zea* (Pearce et al., 2017), *T. ni* (Fu et al., 2018, Talsania et al., 2019), and *P. xylostella* (You et al., 2013). The availability of these genomes should help accelerate our understanding of the effect of baculovirus infections on gene expression of the key host cells used to produce baculoviruses in vitro over the next few years. Certainly, our earlier efforts to optimize HearNPV production in *H. zea* cells using a genome-scale transcriptomics approach to identify host cell genes to up or down regulate were limited by the lack of an annotated *Helicoverpa* genome at the time (Nguyen et al., 2012, 2013a, 2013b, 2016).

Despite a lack of a detailed understanding of exactly how host insect cells activate their antiviral responses when infected by baculoviruses in culture, that has not stopped efforts to improve virus yields in vitro by genetically manipulating host cells. Many reports documenting higher yields are based on the stable expression of viral genes integrated into the host cell genome. These viral genes encode baculovirus or other viral proteins that function to suppress the host cell's innate antiviral pathways. Of these viral immune agonist proteins, inhibitors of apoptosis (programmed cell death) have been the most common types of heterologous proteins expressed in either Sf9 or High-Five cell lines for purposes of improving BEVS yields. Sf9 cells stably expressing viral inhibitors of apoptosis, including P35 from AcMNPV, IAP3 from *H. armigera* NPV (HearNPV), and P-vank-1 from *Campoletis sonorensis* (Cameron) ichnovirus (ScIV), have been documented to produce greater yields of AcMNPV-encoded recombinant proteins upon infection (Lin et al., 2001; Mat Yassim, 2018; Steele et al., 2017).

The expression of a second class of proteins, viral suppressors of RNAi (VSRs), has also been explored in the context of the BEVS. These proteins antagonize the host cell antiviral RNAi response. Two VSR proteins, NSs from tomato spotted wilt virus, and P19 from tombusvirus were shown to improve reporter protein production in Sf9 or High-Five cell lines co-infected with BEVS constructs encoding reporter proteins (de Oliveira et al., 2015; Liu et al., 2015; Oliveira et al., 2011). Furthermore, a recent report documented that the stable expression of the flock house virus B2 VSR protein by Sf9 cells could improve the production of recombinant ß-gal during infections with an AcMNPV BEVS construct (Mat Yassim, 2018). This study also reported that the co-expression of both B2 and HearNPV IAP3 in a stable Sf9 cell line could facilitate an even greater improvement in BEVS yields than that conferred by their individual expression alone.

Another engineering strategy for improving BEVS yields involves the utilization of the host cell RNAi pathway to degrade transcripts of target genes, thereby suppressing their expression (reviewed by Chavez-Pena and Kamen, 2018). Many of these studies have targeted host cell Caspase-1 for silencing by RNAi constructs stably expressed by the host cell genome. Caspase-1 is the primary effector caspase encoded by lepidopteran genomes and is the final node in the apoptosis pathway, which, upon its activation, triggers the execution of cellular apoptosis by cleaving a variety of cellular proteins (Courtiade et al., 2011).

Recent developments in genome editing technologies have gifted cell line engineers with powerful tools for creating gene knockout cells in a targeted manner, including the use of clustered regulatory interspaced short palindromic repeat

(CRISPR)-Cas9 technology (Gaj et al., 2013). This strategy was also applied to generate FDL-knockout Sf9 cell lines that, when used in conjunction with the BEVS, were capable of producing a greater proportion of complex elongated N-glycans than unmodified cells (Mabashi-Asazuma and Jarvis, 2017). CRISPR-Cas9 has also been successfully deployed in a variety of lepidopteran cell lines compatible with the BEVS, including the High-Five (*T. ni*) cell line (Fu et al., 2018; Mabashi-Asazuma and Jarvis, 2017, De Malmanche, 2021), the BmN (*Bombyx mori*) cell line (Izumi et al., 2020; Liu et al., 2014), as well as being used to edit baculovirus genomes in Sf21 cells (Pazmiño-Ibarra et al., 2019).

Despite all these efforts not many stable cell lines that deliver enhanced virus yields are commercially available. However, with better genomic, transcriptomic, and proteomic studies of infected cells identifying better gene targets, particularly for gene knockout studies it seems inevitable that robust improved cell lines will emerge in the near future by engineering the best of the current cell lines available.

13.5.3 Metabolomics of insect cell lines

Detailed studies on tissue culture requirements and metabolism have been performed with the aim of producing higher yields of recombinant proteins, virus-like particles or gene therapy vectors via BEVS. These studies were performed with polyhedrin negative viruses, meaning no OBs were made (Ikonomou et al., 2003; Bernal et al., 2009; Carinhas et al., 2010; Vicente et al., 2011). While the latest fed-batch processes are able to deliver peak yields of recombinant products at cell densities as high as $8-10 \times 10^6$ cells/mL, at least for infected Sf9 cell cells (Chan and Reid, 2016), limited fed-batch studies have been performed with processes producing OB's or with cell lines that have been genetically modified to produce enhanced recombinant product yields (as discussed in Section 13.5.2). Most work generating cell lines with enhanced productivity conduct infections at low cell densities ($1-2 \times 10^6$ cells/mL at most and many are assessed in static cultures), where nutrients are not limiting, and waste products are not inhibitory. If virus yields are substantially increased on a per cell basis in enhanced cell lines then the challenge for fed-batch to deliver these enhanced per cell yields at higher densities ($8-10 \times 10^6$ cells/mL) is increased, particularly if low-cost media and feeds dependent on undefined yeast and meat extracts are to be used (see Section 13.4.1).

It is important to identify what processes may be limiting yields for infections at higher cell densities as this may give insight into what nutrients are limiting at these higher cell densities. For example, it is clear that low per cell recombinant protein and polyhedra yields in high cell density infections of Sf9, Hi5, and Hzea cells are preceded by low per cell yields of viral DNA leading to low mRNA levels (Huynh et al., 2013, 2015a,b). A limitation in virus DNA replication may be the cause of the cell density effect for virus infections. Intracellular levels of dNTPs in cells are normally low and not easy to measure, and viruses that can elevate dNTP levels in infected cells (particularly dTTP levels and particularly in nondividing cells), will produce enhanced virus yields (Ardisson-Araújo et al., 2016; Amie et al., 2013). There is a need to measure dNTP levels in baculovirus-infected insect cells at high cell densities and to use metabolomic approaches to better understand the effect of infections and the cells innate immune response on the regulation of the cell's metabolism (Dolezal et al., 2019; Galenza and Foley, 2019). Virus production and the host cell's immune response will both call on energy and nutrients from the cell and how the cell regulates its metabolism to supply these conflicting needs requires understanding if low-cost culture feeds are to be optimized for baculovirus infections in vitro.

13.5.4 Genetically modified viruses

Various genetic modification strategies to increase virus production or the efficiency of the virus in the field are discussed below. For more information we refer the reader to an extensive review about genetically modified baculoviruses for biocontrol of insect pests published several years ago (Inceoglu et al., 2006). Below we summarize the main developments.

13.5.4.1 Viruses with increased speed of kill

One of the downsides of baculovirus-based bio pesticides is their relatively low speed of kill. A fast-killing natural virus-like SeMNPV still needs 3–5 days to kill its hosts. A nice overview of various attempts to reduce time to death is presented by (Inceoglu et al., 2001). Time to death can be reduced by deleting the viral *egt* gene (Cory et al., 2004). EGT reduces the developmental progress of the larvae by inactivation of the molting hormone ecdysone. Deletion of *egt* also leads to reduced OB yields in larvae, but cell cultures infected with an *egt*-deleted mutant will probably not have this limitation. Speed of kill may also be increased by including a copy of the insect juvenile hormone esterase (JHE) gene (especially a stabilized version, with a longer half-life time) in the viral genome under a viral promoter

(Hammock et al., 1990; Eldridge et al., 1992; Bonning et al., 1997). JHE inactivates juvenile hormone and this leads to the cessation of feeding and the induction of pupation in insect larvae.

Very promising data have been obtained with the incorporation of eukaryotic insect toxin genes, such as the venom from the scorpion *Androctonus. australis* (Linnaeus) (aaIT), which paralyzed the insects and killed them faster (Stewart et al., 1991). Incorporation of the gene for a neurotoxin from the straw itch mite *Pyemotes tritici* (LaGrèze-Fossat and Montagné) in AcMNPV (Txp-1) paralyzed *T. ni* larvae (Tomalski and Miller, 1991) and reduced the time to death by 50%−60% (Burden et al., 2000). The lethal doses in the examples described above were the same as for the wild type viruses. A strong reduction in time to death was also observed with a combination of two cooperating neurotoxins from the scorpion *Leiurus quinquestriatus*, Hemprich and Ehrenberg (Regev et al., 2003). For HearNPV hormonal and toxin-based strategies have been combined by replacing the *egt* gene with the *aaIT* toxin gene, resulting in viruses with a much higher speed of kill (Chen et al., 2000). A major advantage of the paralyzing toxins is the reduced feeding damage caused by the pest insect as they stop feeding well before they succumb to the infection.

13.5.4.2 Viruses with increased infectivity

Attempts to express *Bacillus thuringiensis*, Berliner, (Bt) toxins in baculovirus vectors were successful (Martens et al., 1990; Merryweather et al., 1990), but at first did not significantly improve insecticidal ability. The Bt toxins are normally active at the cell surface of midgut epithelium cells. Thus, the expression of Bt toxins by recombinant baculovirus within the cell did not lead to increased virulence. By making a trypsin-digestible fusion of BT toxin to the AcMNPV polyhedrin protein, the toxin was targeted to OBs and released in the midgut. This resulted in a 100-fold reduction in LD_{50} as well as a 60% reduction in medial survival time of the *P. xylostella* larvae.

The incorporation of enhancin genes also leads to viruses with greater virulence. Enhancins are metaloproteases that were first found in GVs (e.g., Roelvink et al., 1995; Lepore et al., 1996; Kuzio et al., 1999) and later also in several NPVs (Bischoff and Slavicek, 1997; Li et al., 2002; Jakubowska et al., 2006). Enhancins make larvae more susceptible by degrading the peritrophic membrane that lines and protects the insect's gut (Toprak et al., 2012). Deletion of the two enhancin genes from *Lymantria dispar* (Ld) MNPV made the virus 12 fold less potent (Popham et al., 2001). An experiment in which the *M. configurata* (Maco) NPV enhancin gene was incorporated into AcMNPV showed that enhancins may work across species, as the LD50 for *T. ni* larvae was reduced more than 4 times (Li et al., 2003). The lethal time when applied at an LD90 was not affected.

13.5.4.3 Changing the ratio of budded virus and occlusion-derived virus production

Mutagenesis to increase the BV titer may seem to be a solution for low BV titers. However, this may give adverse effects, such as the reduced infectivity in vivo seen for an HearNPV ORF107 deletion mutant (Pan et al., 2007). Whether this is (partly) the consequence of a reduced number of ODVs in the OBs, when the balance is shifted toward BV production, was not analyzed precisely in this case. Using such a mutant for preparing seed stock in order to get high BV titers and applying ORF107 in a transgenic cell during OB production has not been tested yet. In a similar way FP25 K may be supplied in trans by a transgenic cell during OB production, to prevent the FP phenotype. Removing it from the baculovirus will give more BVs when used in a nontransgenic cell.

13.5.4.4 Stabilized genomes

As detailed above, major components that destabilize the genome or the *non-hr* origin of replication and the *fp25k* gene that serves as a transposon insertion hotspot have been identified (see also 4.4.3). Stabilized baculovirus genomes may be achieved by deleting the *non-hr* region, however, whether *non-hr* mutants are as infectious for larvae as the wild type virus needs to be analyzed. Providing the FP25K protein in trans, to prevent transposon insertions in this area, for example, with a transgenic cell line, may be an option, especially when this *fp25k* gene is expressed from an inducible promoter.

13.5.5 Future potential

When the yield of virions per cell in vitro are considered, the potential of genomic/metabolomic studies to improve OB yields is realized. Infected Sf9 cells in culture produce 35,000−40,000 virions/cell for AcMNPV (Rosinski et al., 2002, Matindoost et al., 2014, 2015) and Hzea cells produce a similar number or more for HearNPV (Pedrini et al., 2011; Matindoost et al., 2014, 2015). Most of the virions stay in the nucleus (Rosinski et al., 2002; Pedrini et al., 2011; Matindoost et al., 2015), and in vitro HearNPV OB contain only around 40−45 virions/cell or only 16% of the total

virions produced per cell (Matindoost et al., 2015), so it should be possible to produce up to 600−800 OB/cell. The best yields reported to date are around 400−600 OB/cell for low cell density infections of $0.5-1.0 \times 10^9$ cells/L (Micheloud et al., 2009; Almeida et al., 2010), while the best yields reported for high-density fed-batch infections are 250−300 OB/cell at PCDs of $7-9 \times 10^9$ cells/L (Section 13.3.5.2). An optimistic research objective would be to achieve yields of ≥ 600 OB/cell at peak fed-batch cell densities of 1.0×10^{10} cells/L. If such yields can be achieved for at least a few key viruses (HearNPV, SfMNPV, AgMNPV), then in vitro production of baculoviruses would be an attractive option for industry.

13.6 Conclusion

This chapter is structured as follows. Section 13.1 makes the point that baculoviruses are the most useful natural virus type to commercialize to protect human crops from insect damage and provides an overview of baculoviruses in terms of their structure and lifecycle. Section 13.2 highlights the ongoing market potential for baculoviruses for insect control across many agricultural systems and shows that in vivo based manufacturing of baculoviruses is an effective option to supply these virus products commercially. However, the case is made that in vivo based manufacturing is not perfect and in vitro based manufacturing of baculoviruses could provide some benefits. A key benefit is the ability to produce multiple viruses at scale in a single facility using a relatively generic production process working from frozen master stocks of various cell lines and stable master stocks of baculovirus OBs. Section 13.3 then provides an overview of the current status of in vitro production while Section 13.4 introduces a list of the limitations for bioreactor-based production of wild type baculoviruses. Finally, Section 13.5 highlights research directions aimed at solving the limitations mentioned in Section 13.4.

In terms of economics, the point is made, in the introduction to Section 13.3, that animal cell technology has progressed a lot over the past 40−50 years and can now deliver sophisticated protein products at 1−5 g/L in fed-batch suspension processes at costs that would allow in vitro production of baculoviruses to be competitive with in vivo manufacturing costs. Hence scale-up is not a limitation if we can get OB/L yields of 2−4 g/L through research at a small scale.

Future efforts however still need to deliver on a series of issues that were clearly identified a decade ago by Claus et al., 2012, at the end of their review on the Production of insecticidal baculoviruses in insect cell cultures: potential and limitations. While the issues remain, there is some optimism that progress is being made to solve them. The issues are as follows:

Obviously, a key quality control concern relates to genome stability of the virus in vitro, discussed extensively in Section 13.4.4. Genetic manipulation of the genome to ensure stable production of OBs does not look feasible at this stage given the range of transposable elements that can act to destabilize the virus genome, but further work in this area is justified as discussed in Sections 13.4 and 13.5. The most practical way however to avoid this problem may be to passage the virus for as short a period as possible in culture during the manufacturing process. To ensure this, one option is to produce master and working stocks of the virus as OBs using caterpillars, and then extract and use the ODV form of the virus from these OBs to initiate a bioreactor scale-up process (Reid and Lua, 2005; Almeida et al., 2010). Hence optimization of the ODV extraction process and of ODV infections are important research objectives (Lynn, 2003). The yield and stability of BV of wild type viruses in culture are also nonideal but, in our experience, if BV yields of 10^7 PFU/mL are achieved, and the virus is not stored cold for longer than one week this problem is manageable.

Quality control issues associated with using yeast extracts in the media and feeds for the production process are also of concern. The chemically defined media developed recently for insect manufacturing processes should help in this regard (see Sections 13.4.1 and 13.5.1). This CDM will aid future research. Applying transcriptomic and metabolomics studies to cells grown in a nondefined media limits the potential of such studies to lead to yield increases. It is believed that now that key components have been discovered that allow cells to be grown without the addition of a yeast extract, these components could be supplied independently to cultures as required and it should still be possible to use yeast extracts or other hydrolysates to supply a low-cost source of amino acids for use in insect cell processes. However, these nondefined components will no longer need to be used in a blind manner at high concentrations to supply key undefined trace nutrients.

The economics of in vitro production will be enhanced by approaches that can elevate cell-specific OB yields in fed-batch processes. The key to yield improvements is the successful application of systems biology studies involving genomics, transcriptomics and metabolomics as discussed in Sections 13.5.2 and 13.5.3.

While many challenges remain to make in vitro production of baculoviruses a reality, well-focused funding can potentially solve these challenges. Our knowledge of how viruses interact with cells will improve rapidly now that a few key insect genomes are available (see Section 13.5.2). In vitro baculovirus products would be a big step forward for agriculture, the environment and animal cell suspension culture manufacturing processes.

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Chapter 14

Formulations of entomopathogens as bioinsecticides

Robert Behle[1] **and Tim Birthisel**[2]

[1]*USDA-ARS-NCAUR, Crop Bioprotection Research Unit, Peoria, IL, United States,* [2]*The Andersons Inc., Turf and Specialties Group (retired), Asheville, NC, United States*

14.1 Introduction

Formulation technology is seen as an enabling technology for all pesticides, adding value to the product (Knowles, 2009) and ensuring efficient passage for the active ingredient beginning with the manufacturing step all the way until the application to its final target (Bullock 2020). Microbes have great potential to provide effective management of insect pests when properly developed as biological insecticides. Improving formulations may provide key benefits for microbes to be effective pest control agents in the field (Auld and Morin, 1995). Bioinsecticides have a recognized ecological advantage over conventional broad-spectrum chemical insecticides because specific pest insects can be strategically controlled by biological agents with little or no effect on other plants or animals inhabiting the same environment. Additionally, bioinsecticides generally leave no harmful residues on food crops, alleviating some public concerns relative to food safety. The popularity and use of bioinsecticides continue to expand in markets (Mascarin et al., 2019) as applications of chemical pesticides decrease due to the development of insect resistance to chemical agents, tighter government regulations of chemical insecticides that restrict their application, retail pressure for ecological pest control, and increasing public awareness of the benefits of using biological agents (Van Lenteren, 2012; Ndolo et al., 2019).

Biological agents for control of insects are broadly categorized by Copping and Menn (2000) as (1) living organisms such as predatory insects, parasitoids, nematodes and microorganisms, (2) naturally occurring substances like plant extracts and insect pheromones, and (3) genetically modified plants expressing introduced genes for protection against pests. The United States Environmental Protection Agency (US EPA) defines a biopesticide as one "derived from such natural materials as animal, plant, bacteria, and certain minerals" (EPA, 2020). The posted list of 390 active agents includes plant extracts such as neem oil, fermentation products such as spinosyn, and certain genetic modifications to plants.

In this chapter, we focus on formulations for mass-produced organisms, specifically fungi, bacteria, viruses, and nematodes. As bioinsecticides, these organisms are intended to be applied as an augmentative biological control of insects utilizing an inundative strategy of activity. Inundative activity, as opposed to inoculative activity (Hajek, 2004), is expected to achieve rapid pest control by the mass application of the microbial agent only, with no expectation of control by the subsequent progeny of the microbe. Plant extracts, fermentation products, and insect pheromones also require formulation to be utilized as biopesticides, but formulation considerations for these agents fall more in line with chemical pesticides than with microbial agents. For beneficial microbes intended for classical biological control or for inoculative applications, biopesticide formulation technologies may easily be applied to these pest control strategies.

A formulation consists of a specific combination of ingredients and processes that are applied to an active agent to create a defined commercial product. The diversity of potential microbial agents and target environments combined with the vast range of product forms, processes, and ingredients results in a multitude of potential combinations for the formulation scientist to consider. Potential formulation considerations are further complicated by industrial manufacturing requirements for product handling and worker safety. The task becomes manageable only with a thorough understanding of the entire system from the biology of the microbe through various manufacturing processes, while also

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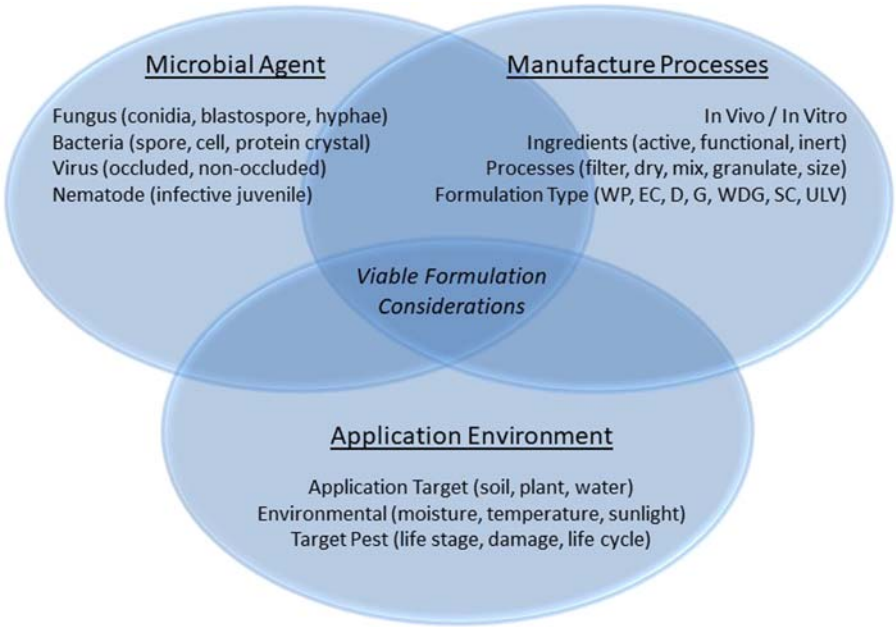

FIGURE 14.1 Spheres of influence depicting formulation considerations while developing microbial-based biological insecticides, which are restricted by specific overlapping knowledge of the microbial agent, manufacture process and application environment.

considering the intended application environment (Fig. 14.1) so that the relevant factors are adequately addressed for successful product development.

14.1.1 Goals and benefits of formulations

"Making useful products usable" was a slogan for the Association of Formulation Chemists and is the primary goal of formulation research for bioinsecticides. Microbial agents can be formulated in different ways to improve their delivery and efficacy (Ash, 2010), and the benefits provided by formulation improvements will contribute to the successful adoption of applied biological control by consumers. More specifically, developing formulations for biological insecticides requires the formulation scientist to select ingredients and processes to address biological considerations relative to the microbe and worker safety, as well as address physical considerations related to handling and application. The proper formulation has the potential to provide numerous benefits for bioinsecticides such as longer storage, easier handling, and greater field efficacy while maintaining the positive ecological attributes inherent to biological pest control.

Often, successful commercial formulations for bioinsecticides are simple formulations that navigate both the microbial production system and the requirements of the end-use application. Such formulations are often protected by trade secrecy provisions in the legal systems of the United States and the breadth of technology involved with formulations is so wide that they are sometimes considered "more art than science." A significant amount of information about formulations is available in scientific literature and through such organizations as (1) the American Society for Testing and Materials International (ASTM, West Conshohocken, PA) Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents which publishes an annual "Special Technical Publications" based on their annual symposia and published ASTM standards; (2) the American Chemical Society (main offices in Washington, D.C. and Columbus, OH), which publishes works based on symposia such as the "AGRO" Division (formerly Division of Pesticide Chemistry); (3) the patent literature which identifies compositions, techniques and ingredients.

Expectations for biological insecticides are generally aligned with expectations for chemical insecticides with regards to ease of use and pest control characteristics. Ease of use relates directly to the physical characteristic of the product, including product form (liquid or dry), storage stability, mixing ability with water for spray application, compatibility as tank mixes with other products, and application method (granule, dust, or spray). Efficacy characteristics refer to the biological impact against the target pest, including pathogen viability, speed of kill, and residual activity after application. Both ease of use and control characteristics are impacted by product formulation, making a selection of the formulation a critical component for product success in the marketplace. Often, the primary goal of the formulation is to allow the application of the biopesticide to fit seamlessly with current chemical application techniques in terms of mixing, application and pest control expectations.

14.1.2 Challenges of microbial pesticides

Years of economical and effective pest control provided by using chemical insecticides have set a high bar of expectations for biological insecticides. These expectations create several notable challenges for bioinsecticide formulations. It is often expected that the formulation will help to fill gaps between the innate ability of the microbe and the expectation of the bioinsecticide. Microbes are particulate and this physical property often presents a problem with mixing in spray tanks. Disease cycles require time to progress, resulting in slower speed-of-kill of the target pest, and can contribute to the perception of low efficacy. Maintaining stability of a biopesticide (microbe viability) during storage is a fundamental requirement and is more difficult than maintaining product stability for a chemical insecticide. Degradation by environmental factors after application shortens residual insecticidal activity, thus requiring more frequent or higher rate applications to maintain pest control. A further constraint on biopesticide development is the high cost of production for many bioinsecticides relative to costs for chemical insecticides, which can limit the monetary margin available for formulation costs. Finally, it can be difficult to maintain organic certification for a product, the desired classification but one that restricts formulation ingredients to those certified by the Organic Materials Review Institute (OMRI). Addressing these are the challenges of the formulation development process.

Within the realm of pesticide formulations, there exists some confusing terminology about adjuvants. The term "*adjuvant*" may refer either to a component of a formulation or to a stand-alone spray-tank additive. As a component of a registered biopesticide formulation, adjuvants are chemicals added to improve the mixing of the formulation components in the product or to improve the mixing of the formulation with water in the spray tank. Selection of the adjuvants added to a formulation is not trivial as the formulation of a biopesticide registered by the EPA is specifically defined and its components cannot be changed without notification and submission of a revised "Confidential Statement of Formula," which could result in a requirement for re-registration of the product. If the formulation change involves ingredients that are already recognized as "lower concern approved inert ingredients," the EPA may allow the change until such time as the periodic re-registration process requires full data support and formal review.

In contrast with formulation components, tank-mixed adjuvants are stand-alone products that are sold separately from biopesticide. These products are intended to be mixed with one or more pesticides to provide some added benefit to the treatment and do not have EPA registration requirements. Benefits may be in the form of drift reduction, improved spreading over the sprayed surface, resistance to wash-off by rain, slower degradation when exposed to sunlight, or feeding stimulation. Disadvantages of using a tank-mix adjuvant include the added cost to the application and the added step to the mixing process. The benefits provided by tank-mixed adjuvants may be difficult to measure or perceive at the user level of the application. Because this chapter covers biopesticide formulations, some of the text may be relevant to "tank-mixed adjuvant formulations" intended for tank mixing specifically with microbial pesticides. Other texts may refer to "adjuvant components" as a specific part of the biopesticide formulation.

A major challenge of microbial pesticides lies in the ability of the biopesticide industry to overcome previous biopesticide failures, which will require the education of consumers and growers to realign expectations for bioinsecticides while positioning products into viable markets. Biopesticide development has been most successful when addressing specific target pests in niche markets and in countries with regulatory, economic, and social conditions that favor biopesticides over chemicals. In reality, many attempts to develop microbes as bioinsecticides for large scale applications fail to achieve all the desired expectations for ease of use and broad pest control. Difficulties with scale-up production and formulation of the microorganism along with inadequate market research have resulted in "orphaned" biopesticides or products that have been withdrawn from the market (Hynes and Boyetchko, 2006). Production costs for many agents are relatively high when compared to chemical agents. In turn, limits funds that can be allocated to formulation ingredients and processes and could result in problems with application and efficacy. Unsuccessful ventures in the marketplace contribute to a lack of consumer confidence when new products become available. Allocating sufficient resources to develop proper formulations should help to minimize the risk of market failure, improve product performance and improve consumer confidence in biopesticides as a viable pest control tactic.

Each microbe tends to impose a unique set of challenges related to the formulation required for application. The uniqueness of each microbial agent impacts the selections among various competing ingredients, processes, and formulation types. For example, baculoviruses are commonly produced using nonsterile in vivo systems. This process results in an active ingredient that consists of homogenized insect cadavers and may contain significant amounts of contaminating bacteria that are expected to be controlled by formulation processes and ingredients. Many fungal bioinsecticides use conidia produced by solid substrate culture, which are hydrophobic and need careful formulation with appropriate surfactants to mix easily with water for spray applications. The bacterium *Bacillus thuringiensis* Berliner is known to deter feeding of target insects and has products that benefit greatly from the addition of ingredients known to stimulate

feeding (Broza et al., 1986; Farrar et al., 2005). For entomopathogenic nematodes, the infective juvenile or dauer has the ability to actively seek a susceptible host but generally requires high moisture content from the time of production through application to the point of host contact. Such differences demonstrate the need for unique formulation solutions among different microbial bioinsecticides.

Formulation research for bioinsecticides faces many challenges but also has the potential to provide significant benefits. Both biological and physical attributes need to be addressed. Some of these attributes are dictated by the end-use application, some by the production system, and some by the formulation process itself.

14.2 Biological considerations

Formulation ingredients and formulation processes address many biological considerations for the microbe as well as the people who handle product from production to end-use application. For the microbe, the choices concerning formulation will impact activity, viability, efficacy, and storage stability characteristics. For the persons handling the product, formulations impact issues of worker safety/exposure, allergenicity, and product contamination.

14.2.1 Biological attributes for the microbe

Many factors, directly and indirectly, affect the efficacy of biopesticides. The formulation scientist must recognize the need to enhance or prevent the inhibition of the intended disease cycle of the pathogen. The formulation should support favorable interactions among the pathogen, host, and, environment to encourage the desired disease development.

14.2.1.1 Activity

The activity of a biopesticide is measured as the amount of the agent required to initiate infection and the length of time to kill the target pest. These characteristics vary widely among pathogens. Pathogens differ in their path of infection, which can be per os, through the host cuticle, through pleural connections of sclerites, or through natural insect openings (anus, spiracles, or oral), which also impacts formulation choices to optimize the activity of each microbe. Formulation choices are determined partly by the physical structure of the pathogen and partly by its mode of action. The structure of baculoviruses, for example, consists of one or more DNA virions encapsulated in crystalline protein occlusion bodies and initiate infection through the digestive tract after being eaten by a susceptible insect. Bacterial also infect the digestive tract of target insects, often aided by bacterial produced protein toxins (Stahly, 1984; Beegle and Yamamoto, 1992; Xu et al., 2006). By contrast, the production of fungal pathogens can be manipulated to form specific structures, including conidia, blastospores, mycelia, or microsclerotia. Fungal activity results not only from direct contact by spray applications but also from infection resulting from exposure to treated substrates (Langewald et al., 1997; Behle, 2006; Douro Kpindou et al., 2011). Conidia and blastospores infect susceptible insects when the spores contact the insect, actively penetrating the integument, whereas mycelia and microsclerotia must subsequently produce the infective conidia or blastospores after application. The infective juvenile nematode enters the susceptible insect usually through the mouth, anus or spiracles, requiring a completely unique set of characteristics to consider for formulation development. These juvenile nematodes require a moist, but not saturated, soil for a week or more following application to allow for their mobility (Gothro and Berry, 2012).

Formulations can optimize activity by improving the chances for the association between the microbe and target pest such as adding feeding stimulants to the formulation to entice pest insects to feed directly on spray residue containing baculoviruses or bacteria, adding oils to improve the adhesion of fungal conidia to insect cuticles, or adding gels to maintain moisture and provide a medium for nematode movement. Each of these examples improves the chance for infection of susceptible insects, thereby benefiting the activity of the bioinsecticide application.

A variety of ingredients can directly improve the activity of specific microbes by decreasing the amount of inoculum required to infect pests or by increasing the speed of kill. Speed of kill of the cattle tick, *Rhipicephalus microplus* (Canestrini) (formerly *Boophilus microplus*), by *Metarhizium anisopliae* (Metschnikoff) Sorokin was improved by the addition of 10% oil emulsion (Leemon and Jonsson, 2008). Additives to stimulate feeding (Sood et al., 2013; Gullickson et al., 2019) and chitinase enzymes have been shown to enhance the activity of bacterial agents when included in formulations (Brar et al., 2006). Chitinase enzymes increase entomotoxicity of *B. thuringiensis* by perforating the peritrophic membrane in the larval midgut, increasing accessibility of the toxin molecule to receptors on the epithelial cells (Kramer and Muthukrishnan, 1997). Sugars, amino acids, and starches can generally stimulate insect feeding, although a few specific compounds can be used to target specific pests. For example, cucurbitacin provides

strong feeding stimulation for corn rootworm beetles (*Diabrotica* spp.) (Arruda et al., 2005). Commercial products based on cucurbitacin have been marketed as tank-mixed adjuvants under names like Coax, Entice, Gusto, Konsume, Mo-Bait, Cidetrak, and Invite to stimulate insects to feed on spray residue (Farrar and Ridgway, 1994; Lopez and Lingren, 1994). Notably for baculoviruses, adding optical brighteners (stilbene derivatives) enhances insecticidal activity, reducing the amount necessary for pest control (Shapiro and Hamm, 1999). The brighteners inhibit apoptosis (cell death) of infected midgut cells, allowing the virus to initiate infection more efficiently (Dougherty et al., 2006).

Although formulations can increase activity, often the goal is simply to maintain conditions to prevent microbe degradation before, during, and after application. For fungal conidia, oil formulations are often desirable for viability, storage stability and residual activity, and provide greater efficacy when compared with aqueous formulations (Kaaya and Hassan, 2000). Formulations address critical factors such as water adsorption and retention necessary for the activity of some fungal agents (Lyn et al., 2010). The activity of aqueous formulations may suffer if the fungal conidia imbibe water too rapidly or initiate metabolic activity prematurely before contacting the target pest. Imbibitional damage of conidia increased with decreasing water activity (a_w = measurement of the energy status of water in a system) of the conidia and decreasing temperature of the immersion water (Faria et al., 2009). In other words, conidia germination decreased when the conidia are dryer and when mixed with colder water. Therefore, it is conceivable that adding ingredients to promote rapid wetting (a desirable physical characteristic) of a wettable powder product could adversely affect conidia when added to a spray tank. When mixed with water for spray applications, conidia may become metabolically active and are susceptible to damage by subsequent drying after application (Leef and Mazur, 1978). Thus, a portion of the spores may be essentially wasted, resulting in reduced activity.

Formulations have the obligation to maintain the maximum activity of the microbe for infecting the target pest. Occasionally, a proper selection of formulation types and beneficial ingredients can improve the activity of the biopesticide, providing improved pest control with the less active agent.

14.2.1.2 Viability and storage stability

Maintaining microbe viability is often considered synonymous with the storage stability of biopesticides. Obviously, loss of microbe viability results in reduced efficacy when applied. Storage stability may also refer to the physical condition of the product irrespective of microbe viability. Maintaining the viability of the pathogen is required for the activity of the formulation and is often more difficult than maintaining the physical characteristics of the formulation. The formulation scientist must recognize that bacteria, fungi, and nematodes need to maintain a minimal level of metabolism to remain viable. (Exceptions are *B. thuringiensis* products that rely on the protein crystal as the active agent rather than viable bacterial spores.)

Assuming production techniques provide technical products with high microbe viability, the formulation processes and ingredients are expected to maintain microbe viability as long as possible during storage. Acceptable storage for microbial agents can be defined as maintaining >90% microbe viability when compared with initial viability, maintaining a predetermined minimal measure of viable microbes per unit of product, or maintaining a minimal level of potency or activity based on a standardized bioassay. Currently, only *B. thuringiensis* products have a standardized bioassay which is used to evaluate product potency (Arora et al., 2006). Fungal products generally claim a minimal concentration of colony-forming units or viable spores. Chemical insecticides are expected to have storage stability for a minimum of two years, but microbial insecticides often measure storage stability in terms of months when stored at room temperatures (near 25°C).

Longer storage of microbial agents in biopesticides may be accomplished by stabilizing cellular membranes, inducing dormancy or slowing metabolic processes of the microbial propagule (Hynes and Boyetchko, 2006). Dormancy is often induced by drying while metabolic processes are slowed by refrigeration. Certain ingredients impact the rate of moisture loss from granules and thus impact the survival of microbes (Lyn et al., 2010). Baculovirus formulations stored frozen or refrigerated maintained activity, but storage at 25°C resulted in significant loss of insecticidal activity and degradation of viral DNA (Lasa et al., 2008).

Formulation types impact the storage stability of microbes (De la Cruz Quiroz et al., 2019). Hydrophobic fungal conidia often remain viable for months when stored in vegetable oils at room temperatures (Kim et al., 2011), which was longer than for dried preparations. Variability among oils has also been noted. *Isaria fumosorosea* Wize SFP-198 conidia remained viable longer when stored in corn oil compared to storage in other vegetable oils (Kim et al., 2011). Dried *B. thuringiensis* products often maintain insecticidal activity for years, even when stored at room temperature. Baculoviruses tend to retain insecticidal activity when stored in aqueous formulations better than in dry formulations and entomopathogenic nematodes require adequate moisture for survival during storage (Leite et al., 2018). Oxidation

can cause a loss of biological activity. However, these losses can be minimized by processing to select the physical state of the formulation (solid vs liquid) and by including chemical additives such as antioxidants or free radical scavengers (Li et al., 1995). In summary, storage stability can be adversely affected by high or low moistures, high temperatures, oxidation, and contamination, which directly degrade the microbial agent.

Changes in the physical characteristics of biopesticide formulations such as caking of powders, separation of liquid components, flocculation of ingredients or settling of suspended particles, often result in poor application and reduced biological activity. These problematic conditions can result from excessive moisture, incompatible ingredients, improper particle size distribution, density differences among ingredients, microbial contamination of the product, or improper processes or handling. Addressing issues caused by the product's physical status tend to follow solutions developed by food, cosmetic, and pharmaceutical industries. Additional information about the physical considerations of formulations is presented later in this chapter. Regardless of biological considerations, adverse alterations to the physical structure of the formulation can impede the proper application of the bioinsecticide resulting in poor pest control.

14.2.1.3 Residual activity

Numerous environmental exposures contribute to the rapid loss of bioinsecticide efficacy in field environments. Ingredients and formulations are available to address many of these adverse conditions. Adverse exposures include exposure to ultraviolet (UV) light energy, excessive or insufficient moisture, wash off by rain, competition by other microbes, and antibiotic leaf chemistries.

Sunlight exposure continues to be considered a major factor that reduces the efficacy of microbial pesticides in the field. Numerous studies have demonstrated the adverse impact of sunlight exposure resulting in rapid loss of microbe viability (Ignoffo et al., 1977; Daoust and Pereira 1986; Inglis et al., 1993) and or loss of insecticidal activity of baculovirus (Ignoffo 1992; McGuire et al., 2000; Wilson et al., 2020). UV-B light energy reduces spore viability of *Bacillus sphaericus* Neide and subsequent larvicidal activity against mosquitoes (Hadapad et al., 2008). For baculoviruses, UV degradation has been linked to damage of the viral DNA (Salamouny et al., 2009). Increasing doses of UV-B exposure to fungal conidia reduced conidia viability and subsequent mortality of exposed aphids (Yao et al., 2010). For entomopathogenic nematodes, exposure to UV-B energy killed *Steinernema kushidai* Mamiya juveniles, decreased the density of their viable symbiotic bacteria (*Xenorhabdus japonicas* Nishimura) and reduced nematode infectivity of cupreous chafer (*Anomala cuprea* Hope) larvae (Fujiie and Yokoyama, 1998).

Microbes have been protected from harmful short-wavelength light energy by adding ingredients to formulations or as tank-mixed adjuvants. Ingredients protect the microbes as a (1) chemical sunscreen by absorbing short-wavelength energy such as optical brighteners, chemical dyes, absorbers (sunscreens) or as a (2) physical sunscreen by reflecting light energy such as clays and titanium dioxide (TiO_2). Ingredients include chromophores like congo red (Hadapad et al., 2011) for aqueous-based formulations. Stilbene-derived optical brighteners that absorb UV energy and convert it to safe visible light wavelengths can be included as components of the biopesticide formulation or added to the spray tank as adjuvants (Shapiro, 1992; Shapiro and Argauer, 1997, 2001; Vail et al., 1999; Reddy et al., 2008). Optical brighteners not only provide UV protection for microbes (Chaparro et al., 2010) but also interact synergistically with many baculoviruses resulting in elevated efficacy (Caballero et al., 2009). For oil formulations, organic compounds commonly used in sunscreens (e.g., oxyl methoxycinnimate) (Inglis et al., 1995) and experimental sunscreens (modified soybean oil known as soyscreen) (Behle et al., 2009) protect the viability of fungal conidia when exposed to natural and simulated sunlight. In novel formulations, UV protection is provided by zinc oxide (ZnO) (Chen et al., 2010) or titanium dioxide (Amar Feldbaum et al., 2021) nanoparticles for *Beauveria bassiana* (Balsamo) Vuillemin conidia and recommends adding 1%−2% to oil formulations made with fungal conidial. For hydrophobic fungal conidia, these oxides have been specifically applied to the spore surface using a Pickering emulsion, where the oxide particles assemble at the oil/water interface to stabilize the emulsion and in this case coat the spores with the protective oxide (Amar Feldbaum et al., 2021).

Moisture affects the biological activity of bioinsecticides in several ways. For nematodes, moisture is required to prevent desiccation and to provide a medium for host searching. Advances in protecting entomopathogenic nematodes from desiccation have been made by adding or incorporating protective gels (Shapiro-Ilan et al., 2016). These gels provide a spry residue with a micro-environment for nematodes to target pests in habitats normally considered inhospitable for nematode survival, such as the above-ground portions of plants. The application of this gel formulation technology offers the opportunity to greatly expand the list of pests targeted by nematodes, including diverse organisms like wood boring insects and ticks (Shapiro-Ilan et al., 2016; Shapiro-Ilan and Goolsby, 2021). Likewise, a certain amount of moisture is necessary for the germination of fungal conidia to initiate infection. However, residual activity

can be adversely affected by excessive moisture as heavy dew or rain that removes the bioinsecticide treatment from the application target. There are several approaches that can reduce the impact of rain. One approach is to add sticking agents as tank mix adjuvants to bioinsecticide applications, while another approach is to include a sticking agent in the formulation. These agents generally consist of natural (carbohydrate, protein or other polymers) (Morales Ramos et al., 2000) or synthetic chemistries (ethoxylated phenoxy alcohols or latex polymers) intended to stick the microbe to the treated surface. The mechanism of protection is provided by molecules that are hydrophobic or that can polymerize or precipitate to form water-insoluble residues to entrap the microbe and reduce wash off.

Leaf exudates are known to adversely affect the residual activity of baculoviruses (Young and Yearian, 1974; Ellerman and Entwistle, 1985; Stevenson et al., 2010). Aldehydes, ketones, carboxylic acids and their derivatives produced by plants have an antibiotic effect on the *B. thuringiensis* (Maksymiuk, 1970). The insecticidal activity of entomopathogens can be further compromised by competing microbes present in the environment. It is probable that encapsulating formulations could protect beneficial microbes by separating them from harmful leaf chemistries and competing microbes. Unfortunately, countering the impact of these adverse conditions has received little attention in the scientific literature to date.

14.2.1.4 Efficacy

Efficacy is the culmination of the bioinsecticide treatment and is often measured in terms of insect control as pest mortality and/or in terms of crop protection. The pathogen, formulation, pest, and environment all impact efficacy. Before application, many contributions of the formulation have already been realized by maintaining the activity and viability of the microbe through processing and storage as well as maintaining the physical properties of the product. Improved efficacy provided by experimental formulations is best evaluated by comparing the efficacy of formulated with the efficacy of unformulated treatments applied under field conditions. Successful formulations are subsequently evaluated commercially by comparing the efficacy of the bioinsecticide treatment with alternative pest control applications. Additional contributions provided by the formulation now focus on the practical aspects of the application including mixing and coverage by spray applications, flowability of granules, and ease of application for dusts to target pest habitats. The formulation can provide built-in benefits by targeting specific pests with feeding stimulants or by extending the residual activity.

14.2.1.5 Integration with production

Developmental research on biopesticides often focuses on production techniques to maximize the yield of infective propagules. It is recognized that proper formulation of these propagules is necessary for storage stability and easy application and is an essential component for product development (Jackson et al., 2010). Formulation development is most efficient when conducted as an integral part of the production. However, the eventual use of the product also imparts a set of considerations necessary for easy application and efficient pest control. Thus, the formulation is the technology that bridges microbe production with pest control and must address the requirements of both the production side and control side of the application. Formulation technology plays a key role in the commercial success of many potential microbial insecticides.

Production and formulation are not uniquely separate processes when developing a bioinsecticide. Many of the processes, such as drying, can be considered as both a step of production and/or a step of the formulation. Thus, greater integration between production and formulation will likely increase the efficiency of the overall process. Whether using either in vitro or in vivo production techniques, the resulting pathogen propagule usually needs to be separated from other production components and concentrated to form a "technical product," which is then processed to the final product form. Fermentation broth, solid media substrate, or insect parts may need to be separated from the pathogen to provide a suitably concentrated technical product. These production components can be removed by screening, filtration, centrifuge, or drying, thus establishing the initial conditions for subsequent steps of the formulation.

At this point, the formulation process has a multitude of options in terms of ingredients and processes that can be performed. Simply drying the microbe may be all that is necessary for a usable product. Many *B. thuringiensis* products are the result of fermentation production, centrifuging to reduce the liquid, and spray drying to form technical material consisting of a fine powder. This technical material may be the final formulation or it may receive additional processing. A carrier such as clay may be added by simply mixing it with the technical material (e.g., using a ribbon blender) to form a dust formulation for homeowner application. Granules may be formed by agglomerating the powder using a pan granulator, fluid bed dryer, or extruder. Granule formulations differ in that some are intended for a direct application while others are intended to be dissolved in water for spray application. Alternatively, the technical powder may be

mixed with aqueous or organic liquids to provide concentrated liquid formulations for application as direct or diluted sprays. Selecting among these possible formulations is often dictated by the conditions of the intended end-use application environment.

14.2.2 Potential hazards

A large amount of basic research is initially directed at identifying, characterizing, and developing production techniques for microbes to be used as biopesticides. Once basic research on a biological control agent is complete and efforts shift to commercial production, worker safety, and quality control need to be addressed relative to the proposed manufacturing processes. Even though entomopathogens are generally considered to pose extremely low health risks (Laird et al., 1990), the active agent, formulation ingredients, and production processes must be evaluated as is legally required for any manufacturing process.

14.2.2.1 Contamination issues

Contamination of biopesticide formulations with unwanted microorganisms during production, storage and application is a concern for both the producer and the general public. Maintaining conditions for viable beneficial microorganisms generally equates to favorable conditions for contaminating microbes. Recognition and quantification of contaminating microbes are important components of quality control of biopesticide production (Jenkins and Grzywacz, 2000). One adverse effect of contamination is the fouling of the product, resulting in either a product that is ineffective (destruction of the active agent) or a product that is impossible to apply (powder caking or emulsion flocculation). A serious problem is the potential to produce human pathogenic microorganisms in the formulation and disseminate them by application to a food crop. Although not a human example, a bioinsecticide application to honeybee hives for the control of Varroa mite resulted in greater death of bees because the biopesticide was contaminated with bacteria (Meikle et al., 2012). Microbial contamination of bioinsecticide products represents a hazard for both the product and the public.

Bioinsecticides can become be contaminated by multiple sources. Most in vitro (liquid fermentation) procedures effectively produce beneficial microbes with minimal contamination. By contrast, in vivo production may have higher amounts of microbial contaminants from the colony insects used in these production systems. Formulation processing steps between production and packaging are often conducted under clean (not sterile) conditions and offer opportunities for additional microbial contamination. Another source of contamination is from nonsterile ingredients added to the formulation. Operating under sterile conditions and adding sterilized ingredients are often cost-prohibitive for the economic processing of microbial insecticides. As a result, the goal of formulation processing is to manage product contamination to comply with regulatory and industry standards. Maintaining clean conditions and ingredients may be sufficient to prevent excessive contamination to develop in the final product. Cold storage and/or drying are commonly used to prevent the proliferation of contaminating microbes. Measuring water activity (a_w) is commonly used as an indicator of product perishability. Maintaining a moisture content below 0.8 a_w generally prevents microbial growth. Controlling pH and adding selective antimicrobial ingredients can also contribute to maintaining contamination at acceptable low levels.

14.2.2.2 Biohazard worker exposure concerns

Hazards to employees are a major concern for all industries including the biopesticide industry. The hazards associated with the production, handling, and application of the microbial agents used in bioinsecticide products are relatively benign. Human safety issues are well documented for fungi (Zimmermann, 2007), bacteria (Peng et al., 2008), and baculoviruses (Burges et al., 1980) used as bioinsecticides. Nematodes go a step further along the safety path, in that nematode products are not required to be registered as pesticides with the US EPA and with many other countries (Ehlers, 2003). These beneficial agents simply do not pose a significant toxicity risk to most workers, although they may induce allergic reactions in susceptible individuals.

Given the safety of these agents, additional concerns about the safety hazards of worker exposure can stem from formulation ingredients. Generally, the ingredients added to formulate bioinsecticides also pose minimal toxicity hazards to humans. Many ingredients are well characterized because of their use in other industries and are generally regarded as safe. Carbohydrates, proteins and vegetable oils can be pulled from food industry applications; organic and inorganic fillers can be adopted from pharmacy or cosmetic industries. The wide availability of ingredients allows for the selection of those with minimal toxicity hazards. Ingredients listed by the United States Food and Drug Administration as Generally Regarded As Safe (GRAS) or those included on List 4A (US EPA) are often selected to be used with

biopesticide formulations. For the bioinsecticide to be certified for organic applications, all ingredients need to be listed in the OMRI database. As for the US EPA registered bioinsecticide products, safety regulations covering works and handlers of pesticides are outlined by the Worker Protection Standard 40 DFR part 170.

14.2.2.3 Allergenicity

One biohazard of worker exposure is the ability of microbes, ingredients and final formulations to induce allergic responses by exposed/susceptible individuals. Allergic responses have been reported for persons exposed to bioinsecticides and insect pathogenic microbes. Reports often focus on the most common bacterial and fungal agents including *B. thuringiensis* (Doekes et al., 2004), *B. bassiana* (Westwood et al., 2006), and *M. anisopliae* (Ward et al., 2009, 2011). Much of the research was conducted using inhalation exposure of laboratory animals (Ward et al., 2009, 2011; Barfod, et al., 2010). Several reports documented responses in human subjects as a result of bioinsecticide application (Petrie et al., 2003; Jensen et al., 2002; Doekes et al., 2004). End users can reduce the possibility of exposure by using personal protection equipment as outlined on product labels when handling, mixing and applying treatments. Formulations with less dust also reduce the risk of inhalation exposure to allergens.

14.2.2.4 Combustion, thermal degradation, dust explosion hazards

The organic nature of bioinsecticide formulations warrants serious consideration of fire hazards. In the United States, the National Fire Protection Association (NFPA) is the leading source of advice and technical information in this area. Labeling and hazard categories are conveyed in Safety Data Sheets (SDS) issued in compliance with the Occupational Safety and Health Act. Some fire hazards may not be obvious. For example, seemingly innocuous materials like powdered milk can carry a weak dust explosion rating by NFPA. This means that when these particles are suspended in air in a confined space and are exposed to a spark caused by static electricity or by a loose metal part making an impact with a bin wall can cause an explosion. Dried and milled grains, sawdust, corncobs and rice hulls have a higher rating class than coal dust and have accounted for explosions in manufacturing plants.

Often an SDS for a given organic material will indicate no special precautions are needed, which may be true on an as-is basis. The data in an SDS includes lower and upper dust explosion limits, flash point temperature, and the auto-ignition temperature, where a material self-ignites in the presence of air. What is not documented on the SDS is that seemingly simple operations such as milling and drying performed upon relatively harmless materials can result in auto-ignition of storage piles, due to common conditions of insulation, compaction, and oxidation. Stored products and ingredients may be degraded or begin to burn as a result of excessive moisture. Heat pockets can develop deep in a product stored in bulk and can move moisture within the pile. Older storage bins may leak rainwater. As a result, auto-ignition may be triggered by the heat of microbial degradation in the case of localized wet conditions. Formulators need to be aware of potentially hazardous conditions associated with scale-up processes and work with fire prevention experts and testing laboratories to prevent disastrous events.

14.3 Physical considerations

Many physical considerations for bioinsecticide formulations are obvious to the observer such as cost and physical form, whereas others are not obvious, for example, specific ingredients and processes required to create the formulation. Currently, production of the active agents is relatively expensive and often limits potential markets to high-value crops, consumer markets, or situations where less expensive control measures are not available (organic production).

14.3.1 Cost

The cost of formulating a bioinsecticide can be determined by simple modeling of the proposed formulation process to include the costs of ingredients, equipment, labor and facilities. The costs relative to the benefits provided by formulation ingredients and processes are an integral part of industrial formulation research. Cost considerations for bioinsecticide formulations tend to be more stringent than for related industries (food, cosmetic, or pharmaceutical industries), predominantly because retail profit margins for production products tend to be less than for consumer products.

Several basic concepts bear mentioned with regards to formulations of bioinsecticides. The unit price of a product increases rapidly, perhaps doubling through each step of a typical distribution chain from the producer through the wholesaler, distributor, and retailer to the consumer. Each small increase at the production/formulation level results in a proportionally greater cost for the consumer. Costs for ingredients tend to garner greater scrutiny than costs for

processing equipment because the unit cost of ingredients is directly related to the units of product made, while the unit costs related to equipment decrease with increased production. In addition to ingredient and equipment costs, formulation related costs can result from added bulk or weight, requirements for refrigerated storage, excessive waste disposal, extended handling time, safety risk, or extra labor requirements. To create a commercially competitive product, the formulation costs must be minimized through the appropriate selection of cost-effective ingredients and processes.

Obviously, low costs are desirable when selecting among ingredients and processes used to formulate microbial pesticides. However, formulations that improve efficacy, extend storage, or lengthen residual activity may allow lower application rates and thus may offset some costs. For example, Chaparro et al. (2010) demonstrated that adding 0.5% optical brightener to a granulovirus treatment for *Tecia solanivora* (Povolny) larvae increased toxicity 50-fold. Adding the brightener not only reduces the amount of active ingredient required and the cost of treatment but could also reduce costs of storage and shipment compared with higher treatment rates of unformulated virus.

A benefit of bioinsecticides is that costs for registration are generally less than costs for registration of chemical insecticides. For example, from 1995 to 2000, new synthetic chemicals required over $185 million and a 10-year development period before reaching the market, compared with a biopesticide that required about $6 million and only 3 years (Kennedy et al., 2007). Government regulatory agencies strive to improve the registration process to favor the adoption of biological control agents (Leahy et al., 2014; Frederiks and Wesseler, 2019). In the United States, the IR-4 project started in 1963 continues to actively support the registration of pesticides specifically for limited acreage crops with a focus on biopesticides (Baron, et al., 2016). In general, regulation agencies tend to support the adoption of biological agents through relaxed registration processes when compared with chemical agents.

14.3.2 Formulation form

Components of a biopesticide formulation are generally categorized into three parts: the active ingredient, carrier and adjuvant (Ash, 2010; Burgess 1998). The active agent discussed in this chapter is the infective form of the microbe, for example, the infective juvenile nematode, fungal propagule, baculovirus occlusion body, or bacterial spore. Unlike chemical insecticides, microbial agents are particulate and are characterized as suspensions in the formulation or spray tank, not solutions or emulsions. The carriers are the components of the formulation that generally serve as inert ingredients used to dilute the active agent with little or no specific benefit. Adjuvant components comprise a wide variety of agents that improve one or more formulation characteristics such as storage (anticaking, humectants, oxygen scavengers), mixing (dispersing or wetting agents), application (viscosity control, spreaders, stickers), or efficacy (protection from degradation by UV or desiccation) of the bioinsecticide.

The three components of the biopesticide products are combined to form liquid or dry formulations. Aqueous and oil-based liquids are often concentrated products that are intended to be applied directly or mixed with additional water for application through spray equipment. Traditional liquid formulations include emulsifiable oils (oil miscible flowable concentrate), suspension concentrates, and oil-based liquids often intended for direct application as an ultra-low volume (ULV) application (Carvalho et al., 2020). Dry formulations include a variety of product formulations from dry granules, (Quiroga, et al., 2011) and briquettes for direct application (Rice et al., 2020) to water-dispersible granules and wettable powders, which are intended to be diluted in water for spray application. Recently, Faria and Wraight (2007) reviewed mycoinseticides and mycoacaracides and defined nine formulation types, adding three to those listed above: bait, contact powder and oil dispersion. These designations cover most formulations used for other microbial pathogens and the characteristics of these formulations are listed in Table 14.1.

Encapsulation of microbial agents has been evaluated to provide a variety of benefits for microbial biopesticides. Two common benefits addressed for application to field-grown plants are moisture retention for nematodes and UV protection for fungi, bacteria, and baculoviruses. The encapsulation could be used to mask microbes known to deter feeding, link synergistic ingredients with the microbe, or protect the microbe from enzymatic degradation on the leaf surface (Stevenson et al., 2010). Many encapsulation technologies have been developed for pharmaceutical, cosmetic, and food industries, and may not be sufficiently economical for adaptation to the less lucrative biopesticide industry. Gouin (2004) reviewed encapsulation technologies used in the food industry, listing spray-drying, spray cooling/chilling, spinning disk and centrifugal coextrusion, extrusion, fluidized bed, coacervation, alginate beads, liposomes, Pickering emulsion, RESS/SAS (supercritical fluids), and inclusion encapsulation as encapsulation techniques. These techniques vary in the physical structure of the encapsulated product. Both true encapsulation (consisting of a shell around a core) and matrix encapsulation (consisting of aggregates of microbes stuck together by a matrix material) can be effective forms of protecting microbes. Propagules can be encapsulated by chemical or physical processes.

TABLE 14.1 Common formulations of biopesticides.

Formulation	Abbreviation	Application
Emuslifiable concentrate	EC	Spray
Suspension concentrate	SC	Spray
Ultra low volume	ULV	Spray
Wettable powder	WP	Spray
Capsule suspension	CS	Spray
Emulsion in water	EW	Spray
Dust	D, P	Dust
Granule	G	Dry
Water dispersable granule	WDG, WG, SG	Spray

TABLE 14.2 Sources for formulation ingredients with reduced risk or organic certification.

Source	Purpose	Website
OMRI	Organic certification	http://www.omri.org/omri-lists
FDA GRAS	Generally regarded as safe	http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm
EPA List 4A	Minimal risk inert ingredients	http://www.epa.gov/opprd001/inerts/section25b_inerts.pdf

The chemical process may include coacervation or forming calcium alginate beads. Physical encapsulation uses processes like spray drying or fluid bed drying to encapsulate propagules.

Specialized formulations often address one or more key characteristics of the microbe or application such as sponges for storage stability of nematodes (Strauch et al., 2000; Chen and Glazer, 2005; Touray et al., 2020), floating briquettes for applications to control mosquito larvae (Brar et al., 2006) or foams intended to expand and fill termite chambers inside structures (Dunlap et al., 2007). These specialized formulations are intended to maximize the potential benefits of the respective microbes as a practical treatment by targeting the unique characteristics of the pest environment. A biopesticide application may be followed by post-treatment irrigation as an aid in penetrating a crop canopy, residue, or mulch material (Anderson et al., 2006). After noting these specialized applications, most general bioinsecticide formulations emulate chemical insecticide formulations by fitting with current consumer application techniques and utilizing available application equipment.

14.3.3 Ingredients

Ingredients used in bioinsecticide formulations are often simple and inexpensive even though considerations for ingredient selection offer vast possibilities. Lists of ingredients with reduced risks or for organic certification can be found at the websites listed in Table 14.2. Ideally, each ingredient included as part of the formulation will add value with minimal cost. Value can be the result of biological benefits to the microbe during processing, handling, storage and application, or can provide physical benefits in terms of easier product handling, mixing, or consumer appeal. Costs associated with ingredients can be indirect; for example, mitigating an explosion hazard when handling dusty materials or legal fees associated with requirements of using regulated materials like antibiotics. Most beneficial properties required of a formulation can be addressed and indirect costs can be avoided by proper ingredient selection.

Scientific literature reports a wide variety of ingredients that have been evaluated in bioinsecticide formulations to provide benefits directly to one or more aspects of the bioinsecticide, many of which have been presented previously in

this chapter. For hydrophobic fungal spores, nonpolar ingredients like oils (Zhang et al., 2020) and waxes (Muniz et al., 2020) tend to be most suitable. ULV application may consist of kerosene-peanut oil combinations (Douro Kpindou et al., 2011), although many "organic" and vegetable oils may be suitable (Kim et al., 2011). Oil formulations often include surfactants to allow the oils to mix with water for aqueous spray applications (Santi et al., 2011). Various surfactants included as a component of an oil formulation have been studied for their direct impact on efficacy (Song et al., 2019), germination and colony growth with results that varied from no impact to significant inhibition of fungal growth (Santos et al., 2012). Larena et al. (2003) demonstrated that fungal conidia survived freeze drying better with skim milk, peptone and sucrose. Similarly, conidia of *B. bassiana* retained greater viability after spray drying when vegetable oil was added to the feed stock (Fig. 14.2). Proper selection among adjuvants is necessary to maintain the viability of the pathogen and to provide for a stable emulsion or suspension of the biopesticide in the spray tank.

However, fewer reports are available concerning ingredients added to address the physical properties of the product formulation. Experimental wettable powder formulations have been reported to include inert powders such as talc, sand, diatomaceous earth, and various clays (Arthurs et al., 2008). Jin et al. (2008) demonstrated that conidia from different fungal strains required surfactants with different hydrophilic−lipophilic balance (HLB) values for optimal wetting time and conidia suspension. Surfactants HLB, a relative measure of a chemical's ability to mix with water or oil. Ingredients with HLB values less than 11 tend to be lipid-soluble and form water in oil emulsions, while those with HLB values greater than 11 tend to be water-soluble and form oil in water emulsions.

Natural ingredients are often used in formulations in order to maintain the environmentally "green" concept associated with bioinsecticides. Polymers are necessary for encapsulation and often include natural carbohydrate and/or protein polymers like starch, sodium alginate, gelatin, acacia gum (Hadapad et al., 2011), and lignin (Tamez-Guerra et al., 2000). Dust formulations require fine powders (often clays or ground corncobs) to dilute microbes for applications. Natural plant extracts have been shown to protect baculovirus from degradation by exposure to UV energy and could be certified for use on organic crops (El-Salamouny et al., 2009). Also, feeding stimulants may be included to target specific pests of cropping systems, using natural components like leaf powders (Rosas-Garcia and De Luna-Santillana, 2006).

14.3.4 Processing

A wide variety of equipment and processes are available to formulate microbes into biopesticide products. Commercial-scale equipment may be specialized for biopesticides or adapted from other industries processing cosmetic, pharmaceutical, or food products. Homogeneous mixing of ingredients is necessary for both liquid and dry formulations. Liquids can be added to large containers and stirred using a variety of available paddles. Dry ingredients can be mixed with a rotary shell, fluidizing paddle, ribbon blender, or a rotating V-mixer. Pasty ingredients can be mixed using an extruder or industrial paddle mixer. These mixing techniques are mostly batch processes, although both liquid and dry metering systems are available to combine ingredients for continuous flow systems. Selecting appropriately sized equipment that is easily cleaned helps streamline the formulation process and minimizes potential contamination of the formulated product.

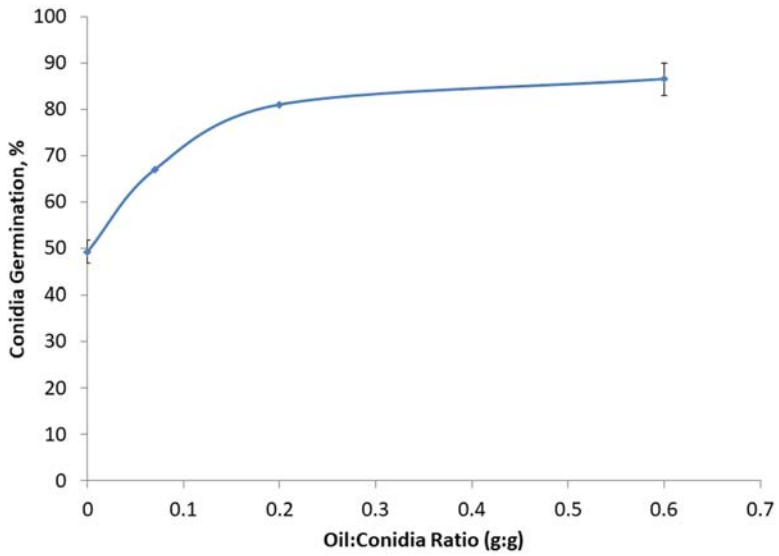

FIGURE 14.2 Example of how an ingredient can benefit microbial agents during processing. Germination of *Beauveria bassiana* conidia increased after spray drying with increasing concentrations of corn oil added to the dryer feed stock. Bars represent standard deviation. *RWB unpublished data.*

Drying is another common and necessary process for many beneficial microbes to slow metabolism and extend storage stability (Lopes and Faria, 2019). Drying equipment comes in many forms including spray driers, ovens, circulating air driers, drum driers, vacuum driers, or freeze driers. Each system has unique features, and not all are suitable for processing viable microbes. For example, freeze dryers have the advantage of being a gentle drying system having a little negative impact on microbe viability. Vacuum driers require sealed vessels and vacuum pumps but allow moisture reduction at milder temperatures than normal sea level pressure. For disadvantages, freeze driers tend to be slower with greater drying expense while operating as a batch system. By contrast, spray driers are more economical to operate with continual flow operation but may reduce microbe viability. Vast amounts of information on dryers, drying systems, capacity, and costs of operation are available from equipment suppliers and such technical information can be accessed through technical publishing houses such as *Powder* and *Bulk Solids* (http://www.powderbulksolids.com/manufacturers/drying-thermal-solids-processing).

Centrifuges, filters, and screens are often used to concentrate microbial propagules by separating the active agent from unnecessary production components such as fermentation broth or insect parts. Liquids and solids can be separated using a centrifuge, in line filtration, rotary-drum vacuum filter, aspirators, or vibrating screen separators. The major advantage of concentrating on the pathogen is to reduce downstream processing costs such as drying. However, this separation produces waste material that may result in disposal issues, so in-line recycling is an important overall system design consideration.

Many production systems yield a product with relatively large particle sizes, which are too large for subsequent processing and/or efficient application. A variety of milling techniques can be used to reduce the particle size to meet physical specifications, though milling may be detrimental to the microbe. For example, Kim and Je (2012) reported on milling of *B. thuringiensis* technical powder and found: (1) that smaller particles had higher control efficacy against diamondback moth [*Plutella zylostella* (L.)] in the laboratory, but not in greenhouse evaluations, (2) that air-jet-milled powder had higher control efficacy than hammer-milled powder, and (3) that excessive processing (second milling) reduced efficacy. This is an example where a relatively simple process such as milling can both positively and negatively impact the biological and physical properties of the final formulation.

Dusty products and their handling are a concern for producers and consumers. Reducing dust improves worker safety and consumer appeal. Various methods are used to reduce dust such as agglomerating small particles into larger granules. In addition to reducing dust, effective granule formulations improve product flow, dispersibility, and application dosing. Granulation can be formed by numerous methods including extrusion, fluid bed agglomeration, or pan granulation. Small and dusty particles can be stuck together using various natural and synthetic chemical polymers.

The benefits of encapsulating microbial agents were discussed previously in this chapter and can be accomplished using a variety of techniques and equipment (Table 14.3). Spray drying is a process of spraying an aqueous suspension of propagules into a chamber of heated air, which rapidly vaporizes the water leaving the solids as a powder to be

TABLE 14.3 Summary of characteristics of potential encapsulation techniques.

Method	Simplicity	Cost	Advantage	Disadvantage
Spray drying	simple	Low	Versatile, continuous, simple	limited to aqueous feed stock, high drying temperatures
Spray chilling/cooling	Simple	Low	hydrophobic process	Untried technology for microbes
Extrusion	Simple	Low	simple continuous process	limited scope, low payload
Fluidized bed	Simple	Medium	Variable shell materials	Batch process
Coacervation	Complex	High	High payloads	Challenging batch process
Spinning disk	Simple	Medium	High payload, high throughput, continuous	Difficult scale-up
Liposome entrapment	Simple	High	High encapsulation efficiency	Costs of process scale-up and delivery form
Rees	Complex	?	No water, mild process	Relatively unexplored technology

collected. Spray drying has long been used for drying and formulating *B. thuringiensis*. An example of a spray-dried formulation of *B. thuringiensis* was reported to contain tapioca starch, sucrose, milk powder, silica fume, polyvinyl alcohol, Tween 20, rice bran, oil, and antifoam solution (Teera-Arunsiri et al., 2003). Kassa et al. (2004) found that spray drying maintained the viability of submerged conidia better than freeze-drying. Also, alternative ingredients such as wastewater and wastewater sludge have been studied as formulation ingredients with spray drying (Adjalle et al., 2011). The final product is a fine powder and the drying process can be controlled to provide one or a few propagules per particle.

Fluid bed drying can be used to remove moisture, agglomerate dust into granules, or apply coatings to particles or granules. The process uses heated-air flow to "fluidize" solid particles in a chamber while applying a liquid spray to the solid substrate. The active agent can be either in the liquid or solid phase. This process is gentler than spray drying. Final products can vary widely in terms of physical attributes such as size, particle density, and particle disintegration.

Encapsulation in calcium alginate (as beads or granules) has been evaluated for a variety of microbial propagules. Gel beads are formed by dripping a mixture containing a dilute alginate solution and the control agent, such as infective juvenile nematodes, into a dilute solution of calcium salt (Chen and Glazer, 2005; Huseein and El-Mahdi, 2019). The calcium ions quickly cross-link alginate to entrap the control agent in the newly formed drop-sized gel. Gel beads can be dried to form hardened granules. A calcium alginate formulation (compared with sodium alginate, gelatin, and acacia gum) provided controlled release of *B. sphaericus* for control of mosquito larvae (Hadapad et al., 2011). The bacterial symbiont, *Photorhabdus luminescens* Thomas and Poinar, of the *Heterorhabditis* spp. nematode was encapsulated in sodium alginate beads and successfully infected *Spodoptera litura* (F.) larvae exposed to a sterile soil environment (Rajagopal et al., 2006).

Coacervation is well known for the encapsulation of ink into small beads then impregnated into a paper to form carbonless copy paper. Coacervantion has been used extensively to encapsulate flavors for the food industry (Gouin, 2004) and has shown promise for encapsulating cells to extend viability (Baruch and Machluf, 2006), but has yet to be reported as a method to encapsulate biopesticides. The process uses dilute solutions of polymers, often carbohydrates and/or proteins, which are deposited around the active ingredient suspended in the mixture. Once capsules are formed, the shells are "hardened" using chemical or enzymatic cross-linking. Capsules can then be separated from the solution and dried. Coacervation has not been reported for microbial bioinsecticide formulations because the process is relatively expensive, complex, and relatively harsh (often using gluteraldehyde to cross-link the shell), three undesirable characteristics (Chilvers and Morris, 1987; Burgess 1990).

Cydia pomonella granulovirus was encapsulated in particles from gas saturated solutions (PGSS) with no loss of biological activity (Pemsel et al., 2010). These particles included ingredients to provide protection from degradation by UV light and phago-stimulants to improve uptake. The PGSS process runs at low temperatures (65°C), utilizing supercritical fluid carbon dioxide. The virus is mixed in a melted fat matrix, then mixed with the supercritical fluid under pressure, and sprayed in a spray tower at ambient conditions. The cooling effect of reduced pressure causes the matrix to solidify and the particles are collected as the carbon dioxide gas is vented. This process has been documented for producing pharmaceuticals, food and food-related products.

14.3.5 Mixing/handling/packaging

Although mixing, handling, and packaging are often associated with marketing, these three physical properties are partly determined by the biopesticide formulation. Mixing refers to the ability of the product to mix with water for application and this characteristic can be measured in terms of wetting time, suspension/settling, or emulsion characteristics. Methods to measure the mixing ability of formulations are published as standards by the American Society for Testing and Materials (ASTM, West Conshohocken, PA). For example, water-dispersible granules should be evaluated for dispersibility, suspension characteristics, particle hardness and dust properties (Al Amin and Bin, 1994). Desirable mixing properties include quick wetting without forming clumps for dry products and forming uniform suspensions (or emulsions) with minimal agitation for dry and liquid products. Effective formulations do not physically separate, which can be described as settling, creaming, flocculation, foaming, or phase separation.

Products with good handling characteristics often go unrecognized either in the factory or by the end-user. Good handling attributes are those that make the product easy to use such as pouring, measuring and mixing. Those with poor handling characteristics are quickly identified by persons working with products. Fine powders often flow poorly and pack tightly in containers from the vibration of processing or shipping. As a result, accurate dispensing and measuring of these powders is more difficult than for "free-flowing" powders. The flow of powders can be improved by adding ingredients such as silica. For liquid products, "thick" or "sticky" describe poor handling properties and can result in

waste of the material remaining in packaging. Propagules that settle or separate in the packing container may not resuspend before being dispensed and thus contribute to inaccurate application and waste by remaining in the package. Bulky or dilute products increase handling costs. Finally, products requiring refrigeration to maintain microbe viability incur additional handling consideration such as refrigerated transport and the associated added expense. Yet, refrigeration may be necessary to maintain propagule viability (Lasa et al., 2008).

Product packaging often provides simple convenience for the shipment of the bioinsecticide from the producer to the consumer. Cardboard cylinders are typical for marketing dust formulations to homeowners, or 5-gallon plastic drums may be suitable for emulsifying oil formulations for farmers. The package must provide sturdy, cost-effective product containment and provide easy dispensing of the formulation. Most common package materials (paper, plastic, glass, cardboard, and metal) are often suitable. Carbo et al. (2019) reported that bottles preserved agent viability better than bags. Additionally, Mascarin et al. (2016) demonstrated longer storage stability of dried fungal blastospores when packaged in sealed containers with sachets containing oxygen and moisture scavengers. Selection among these materials is often directly associated with the form of the formulation.

14.3.6 Consumer esthetics

Consumer esthetics will often reflect mixing, handling, and packaging as described above. The formulation contributes favorably to consumer esthetics when it provides the convenience expected of the product. The amount of convenience varies with the target market. Homeowners operate under the simplest concepts associated with applications of pest controls and generally prefer ready to use products. Trained commercial applicators want highly concentrated products that mix easily for application through spray equipment. Just as the formulation scientist must work with the production system, the scientist must also work with the marketing sector to provide the characteristics necessary for the end-user of the proposed product.

14.3.7 Application

For crops grown under modern agricultural production systems, microbial insecticides have a better chance for adoption when they fit into the current chemical pesticide application systems. If novel equipment or techniques are needed for the application of a biopesticide, then the likelihood for growers to adopt the product for widespread use becomes greatly reduced (Chapple and Bateman, 1997; reported in Bateman, 1999). Most pesticide treatments are applied as sprays, often using equipment specialized for the target crop. Examples include an aerial application for large acreages or tall crops, air blast sprayers for orchard crops, through irrigation systems for row, orchard, and greenhouse crops (Wang et al., 2009) or ground sprayers for vegetable or specialty crops. Properties such as temperature, specific gravity, density, surface tension, viscosity and electrical properties can affect spray characteristics (Solanke and Yadav 2019). Some application systems require specific formulations of the pesticide as spray parameters can affect the efficacy of the treatment. For fungus, higher spray volume and shorter spray intervals improved inoculation of *B. bassiana* conidia onto thrips infesting greenhouse-grown plants, while increasing the concentrations of conidia applied at a constant spray volume had no benefit. The most effective application program for control of flower thrips consisted of multiple applications at shorter than 7-day intervals at the highest rate of the fungus and at the highest application volume (Ugine et al., 2007). Research supports the use of conventional equipment for the application of biopesticides such as hydraulic pumps, selection among nozzle tips, and pumping systems (Chapple et al., 1996; Guimaraes, et al., 2004). Some concerns include loss of nematode viability with repeated passage through centrifugal pumps (Fife et al., 2007; Brusselman et al., 2008), nonuniform discharge of nematodes through drip irrigation (Wang et al., 2009), and potential problems with flow for viscous virus formulations in spray equipment and large drop size resulting in poor distribution and reduced control (Steinke and Akesson, 1993). Spray volume and nozzles affect deposition and infectivity of nematodes applied as foliar sprays on vegetables (Brusselman et al., 2012a,b). Limitations among application techniques for a product are listed on the product label. Application equipment and formulations can directly impact the efficacy of biopesticides, most notably by affecting spray deposition.

Occasionally, unique circumstances may negate potentially effective biopesticide applications. For example, unengorged ticks removed from cattle treated with emulsified conidia died from infection by *M. anisopliae* (Leemon et al., 2008), although there was little effect of this treatment on ticks remaining in the cattle. The conclusion was that an interaction between the environment of the ticks on the cattle with the biopesticide application reduced the efficacy of the treatment. As an alternative, unengorged ticks may be controlled by the application of fungal formulations such as

sprays or granules to vegetative ground cover to infect ticks before they attach to hosts. This research demonstrates the need to direct applications of biopesticides to target the susceptible environment of the pest.

14.4 Additional considerations on formulation

14.4.1 Sources of technologies

Many sources of information concerning formulations have been identified previously in this chapter. Formulations typical to the chemical pesticide industry may not be adequate to fulfill the unique requirements for microbial-based insecticides. Gaugler (1997) correctly suggested that biopesticide formulators establish research linkages with scientists in the food processing and pharmaceutical industries. We further include formulation techniques used by the cosmetic industry. These industries utilize ingredients and techniques that are more adapted to biological systems compared with chemical pesticide industries. As consumer-driven industries, they often develop in-depth formulation research to address aspects of product functionality and consumer appeal.

14.4.2 Legal requirements

Requirements for registration of biopesticides vary among countries around the world. In the United States, obtaining EPA registration as a biopesticide is a major and necessary requirement for all microbial-based active agents [except nematodes which do not require registration in the US or Europe (Ehlers, 2003)]. Regulations for registration of microbial pest control agents in Canada are defined by Pest Management Regulatory Agency regulatory directive DIR 2001−02 and are "essentially harmonized" with data requirements for the US EPA. In Europe, the EU Commission approves active substances, and then EU countries authorize products for use in their territories and ensure compliance with EU rules. Among countries in Central and South America (Cotes 2011), only Colombia has specific regulations for biopesticides established by Decree 1840 of 1994, while regulations in Brazil, Argentina, and Chile follow chemical pesticide requirements.

Meeting label requirements for registration are based on the final formulation to be marketed. One requirement is the identification of the concentration of the active agent, a measurement which differs among active agents. Concentrations of *B. thuringiensis* are identified as International Units based on standard bioassay activity or estimation of protein content (Arora et al., 2006). Conidia counts or CFUs may be listed for fungal products. Virus concentrations are reported as occlusion body (OB or PIB) counts, while nematodes concentrations are viable infective juveniles. The remaining ingredients are often lumped together under the listing as "inert" ingredients on the registration label. These inert ingredients are scrutinized by government agencies and may be subject to restrictions. For example, some commonly used surfactants (nonylphenol ethoxylates) have been banned from use in European markets, prompting a search for more environmentally friendly ingredients (Lowe and Milbradt, 2011).

14.4.3 Current effective formulations

Currently, effective formulations for microbial biopesticides tend to be simple mixtures. For example, baculoviruses use in vivo production techniques resulting in a technical product containing occlusion bodies suspended in a liquid consisting of homogenized insect cadavers. A common and effective formulation is made by the addition of glycerol, which was originally added to prevent the growth of contaminating microbes. The resulting mixture stores well and mixes easily with water for an aqueous application. Similarly, formulations of hydrophobic fungal conidia tend to have good storage stability in oils. Emulsifiers are added to aid with aqueous spray applications. In contrast to oil formulations, the fungal conidia produced using solid substrate techniques are easily formulated as wettable powder formulations by adding dry ingredients to improve the wetting of the hydrophobic spores with water for spray applications. Bacterial based products (predominantly *B. thuringiensis*) are often marketed as sprayable powders. After fermentation, the bacteria are concentrated by centrifuging, mixed with aqueous dispersing agents and spray dried to form a powder. Many commercial bacterial products store well for years, maintaining the viability of spores and insecticidal efficacy of the protein crystal.

14.4.4 Unique applications

Specialized formulations have been successfully developed for specific control situations. Solid controlled-delivery compositions (briquettes) control the release of *B. thuringiensis* or *B. sphaericus* at the surface and/or subsurface

feeding zones for mosquito larvae (Levy et al., 1997). Conidia of *B. bassiana* have been formulated in a wax powder to control varroa mites on honeybees (Meikle et al., 2008). Oil emulsions have been formulated for application to cattle using a motor-driven spray unit for tick control (Leemon et al., 2008). Injections of foaming formulations have been used to coat termite galleries with blastospores of entomopathogenic fungi in living trees (Dunlap et al., 2007). Insect cadavers containing nematodes have been coated to maintain the integrity of the cadaver until being applied to pots for insect control of soil pests (Shapiro-Ilan et al., 2001, 2003, 2010). Wettable granules have been evaluated for simultaneous application with a water spray, to stick granules to the foliage. Baits have been evaluated to induce feeding of housefly adults to initiate infection by the housefly salivary gland hypertrophy virus, which shuts down fly reproduction (Geden, 2012; Baker et al., 2020). These examples of highly specific formulations will not likely result in a grand expansion in the application of bioinsecticides but fill niche pest control opportunities.

A promising new application system that is being developed for use is a combination of "foliar granules" (FGs). These are designed to adhere to aerial parts of plants when applied, the analogous description of sticky "glueballs" is apt. They are applied in a dry state and wetted at the time of application with a wet applied granule system, which is actually a granular applicator which has been equipped with a water spray system that wets the granules after they have left the applicator. Once contacted by water, the FG instantly becomes very sticky via partial dissolution of biopolymers such as proteins or various carbohydrates. The FG products are generally smaller than about 0.5 mm, but this is much larger than the particle size of solids that are sprayed through the typical nozzles used for foliar treatment, so spray drift is virtually eliminated. Since the viscosity of an airborne particle can be much higher than what is sprayed, the adhesion functionality of these wetted granules can allow for positive adhesion without much runoff of the formulation. Tank mix and pumping stability of the FG bioinsecticide is not an issue, nor is the disposal of leftover mixtures and dealing with bioactive liquid spills.

Finally, the application of microbial agents to seeds is increasing in popularity as benefits often go beyond simple insect control. The seed coating process may be as simple as adding a binder, a filler and the inocula to seeds in a cement mixer (Rocha et al., 2019). More sophisticated methods have been reported such as the application of liquid bioplastic formulations for film coating on seeds (Accinelli et al., 2016) and measurements of drying processes of applications to seeds using a spouted bed (Ando et al., 2002). Seed coating has benefited crop production by controlling arthropod pests (Campbell, et al., 2006; Kabaluk and Ericsson, 2007; Pilz et al., 2009; Keyser et al., 2014), reducing infections by plant disease (Keyser et al., 2016; Jaber 2018; Rivas-Franco et al., 2020) and improving plant vigor (Jaber, 2018). Relatively small amounts of inoculum are needed to result in endophytic associations between entomopathogenic fungi and the growing plants (Pena-Pena et al., 2015; Jaber, 2018; Cai et al., 2019) providing an economic advantage when compared with less targeted applications like broadcast sprays. Still, the seed surface represents a uniquely specific environment with will require additional study to determine the precise application rates required and pathogen stability between treatment and planting.

14.5 Conclusions and future of biopesticide formulations

Microbial-based insecticides continue to be promoted worldwide as a favorable alternative to applications of chemical pesticides for pest control. Developing proper formulations is a key component for transforming biological control agents into biopesticides. Just as pest control is best achieved by implementing integrated control tactics, so is the development of a formulation best achieved when based on an integrated research approach that spans from production to final application. Cross-disciplinary education in chemistry, microbiology, and biological pest control could prove most beneficial by specifically training future scientists to tackle integrated biopesticide formulation research. Many physical and biological deficiencies associated with microbial agents can be addressed with proper formulation development and formulations that address these deficiencies will assist in the effective adoption of microbial biopesticide products. Successful commercial biopesticides will foster support for the development of new formulation technologies in the future. Future efforts should continue to study the basic biological, ecological, and epidemiological aspects of beneficial microbes to identify formulations that benefit the viability and efficacy of these microbes as biopesticides. Boyetchko et al. (1999) suggested that the commercial success of bioinsecticides has been limited by adequate biomass scale-up and formulation technologies. Production and formulation tend to go hand-in-hand and continued advancement in both areas is necessary for the successful development of current or newly identified microbes as bioinsecticides. Diligent review of new formulation techniques, processes and ingredients, or those adopted from other industries is necessary to identify those that are suitable for implantation with microbial-based insecticides.

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Chapter 15

Mass production of entomopathogens in less industrialized countries

David Grzywacz[1], Sean Moore[2], Belinda Luke[3], Sevgan Subramanian[4], David Moore[3] and R.J. Rabindra[5]

[1]Agriculture, Health and Environment Department, University of Greenwich, Central Avenue, Chatham Maritime, Kent, United Kingdom, [2]Citrus Research International, Walmer, Port Elizabeth, South Africa, [3]CABI, Bakeham Lane, Egham, Surrey, United Kingdom, [4]Environmental Health Theme, International Centre of Insect Physiology and Ecology, Nairobi, Kenya, [5]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

15.1 Introduction

This chapter focuses on the mass production of entomopathogenic organisms as the active ingredients of biopesticides in parts of the world other than the major industrialized nations. Broadly, this may be viewed as anywhere but the Antipodes, Japan and South Korea, the United States, and Canada, and many countries in Europe. The countries that we will focus on form a heterogeneous group, including many of the poorest countries in the world through the development spectrum up to nations such as China, India, Brazil, and South Africa which are already, or are rapidly becoming, major world economies. Low-intensity agriculture is one common characteristic in these nations, as reflected in the high percentage of the labor force involved. Even here the range is enormous, with the countries referred to in this chapter varying from South Africa with 5.5% of its labor force working in agriculture, to Kenya and India with 37.9% and 42.7%, respectively (Our World in Data, 2020; Central Intelligence Agency, 2020).

Despite, or possibly because of the variations between countries, the less-industrialized countries (LICs) provide a wide range of production examples. What may be considered implicit in the title of the chapter is that low cost, small scale production systems, often with a large workforce, are characteristic of mass production in LIC and many such examples exist in these countries (Haase et al., 2015; El-Wakeil et al., 2019). It is also true that if biopesticides could take their potential market share, demand will be so great that industrial-scale production will be at least a part of production in LIC.

15.2 Issues and opportunities for entomopathogen uptake in less industrialized countries

The control of pests in LIC has always posed a special series of challenges that have created opportunities for entomopathogens (EPs). In tropical countries such as Thailand, where pests have continuous generations, resistance to chemicals develops rapidly so chemical pesticides have a long history of failures (Sukonthabhirom et al., 2011). Many LIC countries also have weak agro-input systems, underdeveloped agricultural research systems and poor agricultural extension advice services. Consequently, farmers, while facing some of the most difficult pests to control, lack adequate resources to assist them. Biological control agents (BCAs) have long been considered as potentially useful tools for pest control in LIC and the wealthier countries. Weak phytosanitary systems in the LICs over the years has also increased the frequency of invasive insects, which often cause catastrophic damage to crop and disrupts the natural regulatory balance in the system.

Amongst these the EPs, such as fungi, viruses, bacteria, and nematodes offer great potential for a number of reasons:

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1. A good biopesticide can offer economic pest control equivalent to that achieved by a chemical pesticide (Langewalde et al., 1999). Their specificity and environmentally benign nature mean they pose much less of a health hazard than the use of potentially toxic chemical pesticides to farmers lacking adequate safety equipment. The residues of EP are recognized to pose no hazard to consumers and produce treated with BCA are not excluded by Maximum Residue Limits legislation/regulations from local or export markets.
2. The ease with which many EP can be produced using low-cost technology has made them attractive options for local production in countries where technical resources are limited. The low-cost technology with which many EP can be produced has made them attractive options for local production in countries where technical resources are limited.
3. Local production of EP is also seen as a way of ensuring costs are lower than that of imported pesticide products, suiting them to the needs of many small farmers able only to afford cheaper pest control technologies.
4. Some key global pests of LIC that have become resistant to chemical pesticides, such as *Heliothis*/*Helicoverpa* spp., *Spodoptera* spp. *Frankliniella occidentalis*, *Bactrocera dorsalis* and diamond back moth (*Plutella xylostella*), have well-known EP. Such EP can provide key tools in the development of insect resistance management (IRM) programs (Marrone, 2007). While some argue the ideal would be to develop biologically-based IPM systems, using no chemicals, this is in some cases not yet practicable. However, incorporating EP into IPM systems is a proven way to overcome chemical pesticide resistance and/or delay its appearance, even though there is a small possibility of resistance developing to certain EP.

The rationale for thus developing EP for IPM is strong and indeed was enacted as a coherent national plan in some countries, for example, India (Rabindra and Grzywacz, 2010).

15.3 Practical constraints for entomopathogen uptake in developing countries

While at a policy level the logic of developing local production of EP in LIC may seem convincing, implementation faces some serious, but not insurmountable, challenges. The size of markets in many LIC is seen as a significant constraint, and apart from Brazil, India, and China where single markets are large. Many LICs have markets with relatively low numbers of customers and or total value. The presently small and fragmented nature of markets for EP in Africa makes it less attractive to set up production unless production can also be for export. Smaller countries can still provide significant markets, for example, Malaysia, Indonesia and the Philippines have enormous areas under oil palm that would be appropriate for EP use. Furthermore, the lack of or weak adoption of quality standards for crop produce destined for local markets in LICs, especially in terms of synthetic pesticide residues does not incentivize the use of biopesticides. The adoption of biopesticides is increasing in crops produced in the LICs but destined for export markets with stringent quality standards and maximum residue levels. Hence the market need exists in LIC, but presently this market niche is largely occupied by chemical pesticides.

In many LIC there is a dearth of knowledge about key pests and the EP available to target them and support potential producers. In sub-Saharan Africa, for example, there has been a serious lack of national funding for agricultural research systems, agriculture in tertiary education institutes and extension services since the early 1990s. This has meant that the technical knowledge base needed to support production and to promote the wider use of EP in farming is lacking. Even where EPs have been registered and are being promoted; weak marketing networks, and poor product regulation in some countries have undermined the market by the proliferation of poor-quality products. Sometimes, a weak understanding of EP and IPM by the farmer, rather than failure of the products themselves, can lead to inadequate control and a loss of consumer confidence in EP. In many LICs agricultural extension systems are weak, and many farmers often rely upon chemical suppliers for advice to guide pest management decisions. This can be a problem where these suppliers are not focused on biopesticides and lack knowledge of EP, but it can be an opportunity if the suppliers take up an interest in supplying EP.

The lack of appropriate infrastructure can also be an important issue for EP supply. Some EP, as with predators and parasitoids whose use is also expanding, require cooling along the supply chain to ensure they are in good condition. Marked improvements in the shelf life of fungal products have ameliorated this constraint to a degree but the improved infrastructure is important. For example, in Africa, outside of horticultural hotspots such as in central and Eastern Kenya or in South Africa where growing produce for export is a mainstay of agriculture, cool chain facilities may be absent. Further diversity of farm sizes (small−medium−large scale farms) in LICs also brings with it the need for diverse packaging for biopesticides to suit the needs. The Kenyan company, Real IPM has developed biopesticide packaging in sachets that is suitable for mixing in individual spray tanks to reach the small holders. Further options to reach small holders through linkages with farmer groups instead of the regular pesticide marketing networks are being developed. The cost of registering an EP pesticide in some countries can be substantial and can deter potential producers

who are largely small to medium-scale entrepreneurs. This issue is compounded in LIC, which can represent small markets, each with a different and separate registration system for pesticides. Consequently, regional harmonization of registration systems would be highly beneficial if not essential if EP products are to become more widely available. There are some positive developments in harmonization of biopesticide registration and use at the regional level, for example, Eastern Africa (East African Community, 2019). Effective implementation of these harmonized guidelines and impacts on the scaled-up availability of the biopesticides needs to be monitored.

In 2009 the EC Regulation No. 1107/2009 brought into operation the "Low-risk substances" category with 11 approvals at the EU level (Marchand, 2018). Eight out of the eleven low-risk substances were microorganisms and most of the substances have no maximal residue limit (MRL) (Marchand, 2018). In Europe, these products are allowed in organic production. By promoting the use of microbial products in LIC it may also open up potentially the organic market allowing the growers to charge a higher premium for their products.

15.4 Production of entomopathogens in less industrialized countries

In most LIC production is largely concerned with entomopathogenic fungi and insect viruses. While there is some production of bacterial insecticides, such as *Bacillus thuringiensis* (Bt) Berliner, these are produced using the same high technology fermenter systems used in industrialized countries. Production of entomopathogenic nematodes (EPN) and protozoa has not yet appeared in any scale in LIC, outside China and the Republic of Korea (Chapter 10 of this volume); in the former case the technological and cost barriers needed to be surmounted have been too great and the lack of a specific market pull means that production has not appeared commercially. There is a renewed interest in the use of EPNs in India, partly because EPNs do not need to be registered. Following successful field trials on the control of the cardamom white grubs, *Basilepta fulvicorne* (Jacoby) with *Heterorhabditis indica* (Poinar), and *Steinernema carpocapsae* (Weiser), with *H. indica*, and *S. carpocapsae*, the Project Directorate of Biological Control, developed an in vivo mass production method using *Galleria mellonella*. larvae and sold the technology to three commercial producers who started selling the EPN formulations. The uptake however will be only for niche areas with high value crops such as the cardamom. In another project, a novel EPN formulation with a shelf life up to 12 months was developed (Kumar et al., 2019). The WP formulation was patented and licensed to at least 16 companies, making these products more widely available to farmers. These products are now used in an estimated 20,000 ha in several states for the control of white grubs and other soil pests in areca nut, banana, brinjal, cardamom, groundnut, and sugarcane. Another example is the development of a gel formulation of *Steinernema thermophilum* developed. The product also has an extended shelf life and is active against white grubs, termites, and many lepidopteran pests, including lepidopteran eggs (Ganguly and Rathour, 2014). DuduTech in Kenya is listed as a manufacturer of three EPN products, based on *Heterorhabditis bacteriophora*, *Steinernema feltiae* and *Phasmarhabditis hermaphrodita* (Dannon et al., 2020). No detailed information is available on the production technology used, but Rovesti et al. (2007) imply that liquid fermentation is used. Production at this time was reported to be only around 10 billion infective juveniles (IJs) per week and described as semicommercial. An in vitro liquid production technique for EPN has been developed by a South African company, Nemabio in collaboration with its academic partner, Stellenbosch University. Based on a series of successful laboratory and field trials against a range of fruit crop pests (Hatting et al., 2019), the objective is to mass rear indigenous EPN species and isolates, a key focus of this EPN applied research is to develop low-cost production media and systems (Ramakuwela et al., 2016; Kotchofa and Baimey, 2019). However, several regulatory hurdles must still be cleared, requiring substantial further investment, which is still pending. In South America, the development of EPN is well advanced with many useful lessons and technological innovations, though the tendency to publish in non-English publications has restricted the dissemination of this research (San-Blas et al., 2019).

Mycoinsecticides (biopesticides utilizing entomopathogenic fungi) are commonly produced in LIC; in this chapter, the emphasis is on low to intermediate technology levels of their production. This is partly to reduce overlap with Jaronski (Chapter 11 of this volume) and partly because industrial-scale production occurring in China, for example, is bedevilled with the same secrecy as in other parts of the world. Although some public producers are quite open with their methodologies, dissemination is often through gray literature which is difficult to access.

The production of insect viruses has almost exclusively focussed on baculoviruses; here again, there are few detailed publications on the various commercial production systems; though, there are some better-documented cases such as the *Anticarsia gemmatalis* NPV (Moscardi, 1999, 2007; Moscardi et al., 2011). Many producers closely guard their production methodologies which they see as commercially important intellectual property, which severely limits the accessibility of information.

15.5 Production of entomopathogenic fungi

In the late 1950s and from the 1960s, China and Brazil (then developing countries) pioneered the extensive use of fungi (Prior, 1989), with *Metarhizium anisopliae* Sorokin being used over many thousands of hectares, to control the sugarcane froghopper *Mahanarva posticata* in Brazilian and Chinese sugarcane, using *Beauveria bassiana* against *Ostrinia nubilalis* also on a very large scale. The increasing wealth and technological sophistication of these nations could lead to transformations in the production and use of mycoinsecticides. Li et al. (2010) describe the history and present status of the use of mycoinsecticides in both these countries while Feng et al. (1994) described in some detail the production of *B. bassiana* in China. Jaronski (2014) provide a useful overview of entomopathogenic fungal mass production over the last 30 years. In India, *M. anisopliae*, *B. bassiana Hirsutella thompsoni*, *Isaria fomosorosea*, and *Lecanicillium lecanii* are produced by several companies and have been registered (taxonomic advances may make some species names tenuous; registered names will be maintained here although *Metarhizium* spp. may prove to be more accurate in many situations). Poor quality control, short shelf life and poor persistence in the field have been major constraints in enhancing the use of these fungi. Kumar et al. (2019) and Jaronski and Mascarin (2017) provide information on the importance of not only cost-effective production methods with good quality spores but also the importance of stable formulations.

Despite a number of publications, there is little real detail about mass production methodologies at the industrial scale necessary to meet major markets. There is a vast amount of gray literature; but these mostly describe minor variations on well-established themes, types of solid substrate, methods of sterilizing or types of containers used and are often difficult to source. The more modern LIC producers tend to keep at least some of their production processes secret. However, our impression is that whereas 20 years ago the focus was on quantity of production, more recently it has been acknowledged that product quality is also vital.

Brazil, India, and China have long and extensive programmes to develop EP as biopesticides and a range of publications demonstrate the extent of interest (Li et al., 2010; Koul, 2011; Leng et al., 2011; Lacey et al., 2015; Kumar et al., 2019; Tripathi et al., 2020; Mishra et al., 2020) and the range of local biopesticide production systems that have been developed. de Faria and Wraight (2007) assembled a comprehensive list of 171 mycoinsecticides and mycoacaricides developed since the 1960s; 15% were no longer available and a further 10% were of uncertain status. South American Institutions and companies were responsible for the development of around 43% of the products.

Roberts and St Leger (2004) bemoan the fact that there is relatively little detailed information on specific mass production facilities in general, and almost none from commercial companies. However noncommercial organizations fill some of this gap (Alves and Pereira, 1989; Feng et al., 1994; Jaronski (this volume); Jenkins and Goettel, 1997; Jenkins et al., 1998; Cherry et al., 1999; Jaronski and Mascarin, 2017). An excellent, and charmingly quirky, description of production processes is given by Grace and Jaronski (2005). This gives a clear, step-by-step account of mass production techniques; but most importantly, it emphasizes the need to view each isolate individually. Jenkins et al. (1998) and Cherry et al. (1999) detail the mass production of *Metarhizium acridum* at the International Institute for Tropical Agriculture (IITA) in Benin, West Africa, during the LUBILOSA program, which was aimed at developing an alternative to chemical insecticides for use against locust plagues. Over a 13-year period the program developed the commercial mycoinsecticide Green Muscle (Lomer et al., 2001; Moore, 2008). To supply conidia for major field trials a mass production facility was built at IITA, becoming operational toward the end of 1996 and producing through into 1998, allowing economic assessments to be made.

15.5.1 The LUBILOSA system

The production capacity adapted systems are used widely in Brazil and China, but the basic principles were used much earlier. Rorer (1910, 1913) gives details of production using a cabinet method, this being before the age of plastic bags. The LUBILOSA method was based on low capital investment and high labor inputs and was robust, simple and appropriate to developing country situations (Cherry et al., 1999) while still capable of producing high-quality fungal material. The locust control project required the complete separation of conidia from the substrate to allow formulation for controlled droplet application, making the LUBILOSA system somewhat more complicated than some other mass production systems may need to be. In summary, the process required the maintenance of the isolate stock (achieved by good storage of material passaged through the desert locust at six-monthly intervals), which was used to inoculate sterile liquid medium prepared from cheap and locally available materials such as waste brewer's yeast and sugar (Fig. 15.1). The liquid inoculum was incubated for a few days and then added to bags of sterile rice which, in turn, was incubated for around 10−12 days. These bags were cut open; the conidiated rice was dried to around 20% moisture;

FIGURE 15.1 Flasks of *Metarhizium acridum* in liquid media on shakers at the IITA facility in Benin.

then the conidia were harvested and dried to 5% or less, before packing in plastic-lined foil sachets. Production capacity was around 300−350 kg of conidia per year, allowing the treatment of about 7000 ha. The system is highly flexible, and many process variations can be used. These may include the local availability of substrates, nutrients and equipment, energy and labor costs and, critically, the fungal isolate(s) used and pest systems in which the mycoinsecticide is to be used. One common characteristic of relatively low-intensity mass production units is that, usually, there is loose control over certain production parameters such as temperature for substrate incubation and relative humidity and temperature for the drying processes. In addition, the exact composition of the substrate used may vary over time, from harvest onwards and perhaps from the cultivar used.

The LUBILOSA process resulted in major variations in production, especially yield, apparently occurring in a cyclical trend. Temperature and incubation period were key factors, but they explained less than 40% of the yield variation (Cherry et al., 1999); the nutritional quality of the rice substrate was also considered likely to be important, but this was not examined in depth. Even minor variations can significantly alter spore characteristics as represented by shelf life or thermal tolerance (Hong et al., 2001; McClatchie et al., 1994) and be of considerable importance to quality. Consequently, with low-intensity production, quite major variations in yield should be expected over time and variations in the intrinsic quality of the spores are also inevitable. This variation is not unique to loosely controlled systems as even systems with accurate process control over aspects such as aeration, humidity and temperature can show considerable variation (Bradley et al., 1992).

15.5.2 The Caroni system

Metarhizium anisopliae was developed for the management of the sugar cane froghopper, *Aeneolamia varia saccharina* (Distant), a serious wet season pest of sugar cane in Trinidad. This project was conducted at the Caroni Research and

FIGURE 15.2 Inoculated rice incubating in plastic containers at the IITA facility in Benin.

Development Division of the national sugar cane research institute in Trinidad and Tobago and described in Jenkins et al. (2007). The production facility was located in the central region of Trinidad and Tobago. The main plant equipment included locally built two steam-reliant type autoclaves, a diesel-fueled boiler with an accompanying water treatment system, incubators, clean benches, microscopes, an in-house designed and built spore-dryer and a cyclone harvester.

The production process was based on the LUBILOSA system and included the discrete activities of sterilization, inoculation, incubation under strain-specific environmental conditions, drying, cyclone extraction of conidia, packaging, storage, and distribution. However, the scale of the operation was quite different with a handling capacity of 250 kg/batch and a weekly throughput of 3000 kg. The production unit operated in 2−12-hour shifts with each shift consisting of 20 persons including supervisory, technical and skilled labor.

A combination of unpolished rice and tap water was packaged in autoclavable plastic bags manufactured within the facility. The bags were partially sealed, stacked in the autoclave and sterilized for 1 hour. About 3−5-day-old-liquid inoculum, produced in yeast and glucose, was used to inoculate the sterilized rice within the plastic bags. The bags of inoculated rice were shaken to thoroughly mix the inoculum amongst the rice and then resealed with micropore tape to allow airflow. For conidia production, the inoculated rice was incubated for 14 days in stacked plastic containers (Fig. 15.2) during which the bags were intermittently shaken to break up clumps and encourage aeration (some isolates do not grow well if the bags are disturbed, so this stage would be omitted). For harvesting, one face of the bag was cut away and the bags were placed in a cool dehumidified room for 48 hours. Conidia were extracted with an industrial cyclone extractor (http://www.mycoharvester.info/) and placed in the dryer for further drying. The conidia were then sealed in plastic bags and stored at 4°C. Although not optimal for storage, plastic bags being neither totally water nor air proof, this was appropriate for a system where the conidia were used promptly. At the conclusion of the 10-year period of R&D (1991−2001), the annual production increased to 1500−2000 kg of spore powder that showed a high level of viability, virulence and purity, and was sufficient for treating around 40,000 ha. The rice substrate yielded an average of 45 g of dry conidia/kg of rice with an end product concentration of 5×10^{10} conidia/g and very high-quality conidia were produced. Research then showed that *A. varia saccharina* could be controlled using preconidiated rice taken from the plastic bags and applied to the base of sugarcane plants; conidiation occurred in the field. This effectively halved the time period required for production. This project ended with the privatization of sugarcane production in Trinidad and Tobago.

15.6 Additional examples from other countries

15.6.1 China

Although China can now by no means be described as an LIC, useful lessons can be drawn from its rapid development of EP production, which has evolved rapidly from early low technology production systems to the current state of the art industrial biotechnology processes used to produce commercial EP products. The development of fungal biopesticides was strongly supported in China in terms of, research, production, and use programmes by local and central governments (Wang and Li, 2010). Details of production methods are given in Li et al. (2010) and Feng et al. (1994). Modern and sophisticated production facilities have begun operating, reflecting the true industrial nature required for mass use over the scale required.

15.6.2 India

Fifteen species and subspecies of microbes have been registered as biopesticide products for pest control by 2016. Based on these species, 970 registered biopesticide products manufactured by 466 companies are available. The products belong to various groups; such as, entomopathogenic bacteria (*B. thuringiensis, Lysinnibacillus sphaericus*), entomopathogenic fungi for insects and nematodes (*B. bassiana, M. anisopliae, Verticillium lecanii, V. chlamidosporium H. thompsonii* and *P. lilacinus*), baculoviruses (*Spodoptera litura* nucleopolyhedrovirus; *Helicoverpa armigera* nucleopolyhedrovirus) and crop disease antagonists (*Pseudomonas fluroescens, Trichoderma harzianum, T. viride, Ampelomyces quisqualis, B. subtilis*) (Srinivasan et al., 2019; Mohan et al., 2017; Wickramaarachchi et al., 2017). Beyond these, commercial products based on entomopathogenic nematodes do not require registration. and there are commercial products based on them.

In India, there are 361 biocontrol laboratories, which includes 179 private sector laboratories; while the rest are government laboratories; however, only very few of these labs are involved in large-scale production of biopesticides (Mishra et al., 2020). Biopesticides consumption has increased from 123 MT in 1994−95 to 8110 MT in 2011−12. However, this declined to 5152 MT in 2014 and further has grown by 40% from then to 2018−19. Fungal (66%), followed by bacterial (29%), and viral (4%) are the types of biopesticides consumed. Among the fungal biopesticides, *Trichoderma* spp. (56%) dominates the market, followed by *B. bassiana* (17%), *Verticillium* spp. (15%), *M. anisopliae P. lilacinus* (6%) and *H. thompsonii* (0.15%). It is estimated that more than 70% of the locally produced biopesticides are from the public sector laboratories and the rest are produced by private companies. Lack of experience in production, poor quality control, poor shelf life, and narrow host range are key technological barriers that need to be addressed urgently to significantly enhance consumer faith and adoption, which is also low largely (Mishra et al., 2020). The efficacy of biopesticides is a function of effective strains and high-quality formulations. However, technological shortcomings in the biopesticide sector of India include both; weak bioprospecting and curation of strains, the need for improvements in formulation and fermentation technology to ensure longer shelf life, quality, and efficacy. It is encouraging to note that novel nanotechnology-based, nanoencapsulation, and nanoemulsion formulations are under development (Koul, 2019; Mishra et al., 2020).

15.6.3 Brazil

Brazil has 82 microbial pesticide registered products with 60% of those based on fungi, 29% bacteria, 10% baculoviruses, and 1% nematodes (Mascarin et al., 2019). *M. anisopliae* is still used against spittlebugs in sugarcane (Li et al., 2010) and represents the largest mycoinsecticides control program worldwide (Mascarin et al., 2019). Additionally, *B. bassiana* is used to control the coffee berry borer and whitefly in over 1 million ha (van Lenteren et al., 2018). This growth is still ongoing with several global and domestic companies looking at expanding their markets in Brazil (van Lenteren et al., 2018).

15.6.4 Cuba

In 1982 Integrated Pest Management was adopted by the Cuban state as official policy for pest control and a small number of industrial scale production centers were established (Henderson and Sinfontes, 2010). These centers, by the late 1990s, had fermenters ranging from 5 to 500 L capacity in which they produced Bt and entomopathogenic fungi; although solid-state production was preferred for fungi (D. Moore unpublished). Biological control production is centered on CREEs (Centros de Reproducción de Entomófagos y Entomopatógenos), which are centers for mass production of both macro and microbial agents. Consuegra (2004) states that in the 10-year period 1990−99, around 5650 tons of EPs were produced, with *B. thuringiensis* making up 60% of the production and *B. bassiana* 30% with the remainder being *Lecanicillium, Metarhizium* and *Isaria* species. Even the lower figure suggests a production of 1.5 tons of *B. bassiana* per year from each of the 130 EP-producing CREEs. It is unlikely that these levels were achieved even if these figures include the solid substrate (D. Moore unpublished). Certified microbial strains are provided to CREES by WAPROSAV, a network of Provincial Plant Health Laboratories (Henderson and Sinfontes, 2010) who also inspect each CREE 3−4 times a year (D. Moore, unpublished). Production methods vary in CREES, especially in terms of solid substrate used, which reflects the local availability of rice, coffee, sugarcane, or maize (Henderson and Sinfontes, 2010; D. Moore unpublished). A range of isolates are produced and used over the country, as appropriate for local pests and mycoinsecticides are used as standalone controls or in conjunction with other biorational methods such as pheromones (Henderson and Sinfontes, 2010).

15.6.5 Honduras

The Fundación Hondureña de Investigación Agrícola (FHIA) produces a product consisting of conidia formed in rice, dried and sold to farmers who wash the conidia off and apply them as a water spray (J.T. Kabaluk, Pers. Comm.); with basic personal protective equipment, this is quite safe (though buying a liquid formulation would remove any respiratory hazard present in the powder form). Production was based on standard texts (Jenkins et al., 1998; Grace and Jaronski, 2005), but the need for customizing production according to isolate was emphasized. Consequently, the need for their own production manual was stressed. Tap water was found to be better than purified water for production (this could well be a very localized phenomenon, as tap water is highly variable, but demonstrates that customizing production is very important). Suggestions for improving production from J.T. Kabaluk (Personal Communication) included giving the laboratory coordinator experience in an established mycoinsecticide laboratory, obtaining an autoclave for sterilization, and developing a dry product with long shelf life.

15.6.6 Kenya and South Africa

Microbial pesticides in Africa are mainly available in areas of strong horticultural production, especially for export, with Kenya and South Africa being the leading champions.

In Kenya, the use of biopesticides for integrated pest management in high-value horticultural production systems has been increasing over the years, with entomopathogenic fungi, *B. thuringiensis, H. armigera* NPV, and entomopathogenic nematodes among the biopesticides used by export horticultural farms. Among the major private sector, biopesticide producers in sub-Saharan Africa, Real IPM Kenya ltd (specializing in entomopathogenic fungi-based products; plant endophytes, and predatory mites); Dudutech (specializing in entomopathogenic fungi, Entomopathogenic nematodes and macrobials) and Kenya Biologics Ltd. (specializing in *H. armigera* nucleopolyhedrovirus and insect attractants) are based in Kenya. The use of biopesticides produced by these private sectors is significantly increasing over the years; for instance, the use of four entomopathogenic fungi-based products produced Real IPM, which has increased over threefolds between 2015 and 2019 (Akutse et al., 2020). Beyond Kenya, these biopesticides are registered for use in nine sub-Saharan African countries. Within the framework of the harmonized biopesticide registration protocols approved by the East African Community, registration of biopesticide products for fall armyworm management is being fast-tracked (Akutse et al., 2020).

In South Africa, of the 31 biopesticide products registered for use, seven of them are locally produced, and are mainly based on *B. bassiana*. with their use growing, particularly in South Africa (Hatting et al., 2019). Products may be of indigenous or exotic isolates, the regulatory environment being quite pragmatic, but fungi production is generally achieved by conventional methods. However, some liquid formulations involve innovative techniques and some solid substrate techniques have employed clay beads. The main commercial producers in South Africa are currently BASF (previously Becker Underwood and BCP), and Plant Health Products, both in South Africa (Hatting et al., 2019). Beyond Kenya and South Africa, local production of a biopesticide is also extensively undertaken in Morocco by Eléphant Vert (Hatting et al., 2019), who are currently the only licensed producers of locust biopesticides.

Although there are many descriptions of the mass production of entomopathogenic fungi in LIC, the major references cited in this chapter (and references therein) cover most of the issues. Jenkins et al. (1998) and Cherry et al. (1999) give possibly the most detailed accounts of mass production in commercial-scale situations. Jenkins et al. (2007) illustrate scaling up while Grace and Jaronski (2005) emphasize the central truth that systems must adapt to the isolate, not the other way round.

15.7 Other systems

Some innovative schemes use less common techniques. Mass production inside coconuts exploits the sterility and complex nutritional nature of coconut water. Difficulties involve maintaining sterility however good production is possible, but the logistics make it impractical for most circumstances. Other substrates have been used as mentioned above. These have included waste from aquaculture and poultry units along with the far more conventional waste from breweries. For example, Torres et al. (1993), working in Peru, used wheat, pearl barley, moss (also in combination with prawn residues) coffee residues (postpulping), sugar cane residue, maize and brewery residue. Torres et al. (1993) refer to coworkers focusing on residues of local crops of another area in Peru, such as inflorescences and grains of *Chenopodium quinoa* and *Amaranthus caudatus*, as well as husks of barley oats and wheat.

A rare attempt at low-intensity production in liquid culture is described by Srikanth and Singaravelu (2011). *B. brongniartii* is produced in dilute molasses broth medium in autoclavable PVC bottles or glass jars. The fungal mats

that grow on the surface are harvested after about three weeks. Another system using *B. brongniartii* involved collecting the larvae of the Andean potato weevil, *Premnotrypes* spp., and placing them in fungus-infected soil (Cisneros and Vera, 2001). A short while later, infected insects and soil were applied to the soil of local potato stores. This is a very niche-oriented method and could well be one of the few examples of truly local production that could be sustainable. One interesting feature was that the fungus survived for long periods in dried cadavers.

Some success has been achieved at the experimental level with producing conidia on nonnutrient substrates (this assumes that most substrates supply a valuable nutrient source). Yields of up to 3×10^8 conidia per square centimeter of cloth were harvested from absorbent cellulose cloth inoculated with a culture containing brewer's yeast and sucrose (Jenkins and Goettel, 1997).

15.8 Mass production of baculoviruses

The production of baculovirus (BV) has been reported as established in a number of LIC (Table 15.1). The commercial production of baculoviruses has been entirely through in vivo production in insect hosts. While in vitro production has

TABLE 15.1 Production of BV in less industrialized countries either on commercial scale or pilot production.[a]

BV species	Countries with commercial production	Countries with pilot or NGO production
Adoxophyes orana GV	Thailand	
Amsacta albistriga NPV		India
Anticarsia gemmatalis MNPV	Brazil, Mexico	Paraguay, Bolivia, Argentina
Autographica californica MNPV	El Salvador, Guatemala	
Autographa californica mNPV + *Spodoptera albula* NPV	Guatamala	
Condylorrhiza vestigialis NPV		Brazil
Cryptophlebia leucotreta GV	South Africa	
Cryptophlebia peltastica NPV		South Africa
Cydia pomonella GV	Argentina	Chile, South Africa
Erynnyis ello GV	Brazil	Columbia
Helicoverpa armigera NPV	India, Kenya, Thailand, Brazil	Vietnam, South Africa, Afghanistan, Bangladesh
Helicoverpa zea NPV	Mexico, Brazil	
Homona magnamina GV	Thailand	
Hyblea puera NPV		India
Phthorimaea operculella GV	Bolivia, Peru	Venezuela
Phthorimaea operculella GV + *Bacillus thuringiensis*		Bolivia, Ecuador
Plutella xylostella GV		Kenya
Spodoptera albula mNPV	El Salvador, Guatemala	
Spodoptera exempta NPV	Tanzania	
Spodoptera exigua NPV	Mexico, Thailand	Nicaragua
Spodoptera frugiperda mNPV	Brazil	Nicaragua
Spodoptera litura NPV	India, Thailand	Bangladesh
Spodoptera sunia NPV		Guatemala

[a]Key sources, CPL business consultants (2010), Haase et al. (2015), Lacey et al. (2015), Wickramaarachchi et al. (2017), Srinivasan et al. (2019), Sosa-Gómez et al. (2020), Tripathi et al. (2020).

been intensively researched and is seen by many as a key technology to expand BV use, large scale production systems for BV have yet to be developed enough to be commercially viable (Granados et al., 2007; Szewcyk et al., 2006; Moscardi et al., 2011; Chapter 13 of this volume). A significant difference between BV production in OECD and LIC is that mechanization in production is used widely in Europe and USA by producers reducing the need for expensive labor. In LIC such labor costs are much lower and capital often harder to access, so labor intensive production systems are more common and more financially viable, especially in small start biopesticide companies that have appeared in countries such as India. However, this also brings in the challenge for small and medium producers of addressing significant contaminations and quality issues with nonautomated production systems.

In vivo production relies on the controlled infection of susceptible hosts at the optimal growth stage and dose of inoculum and the subsequent rearing of these at optimal temperature conditions to allow optimal multiplication of the BV (Kumar et al., 2005; Subramanian et al., 2006). The insects are then harvested, and the infective occlusion bodies extracted. This in vivo system is cost-effective because BV systemically infects a high proportion of the host cells so that the production of viral occlusion bodies can reach 15% of the dried weight of the cadavers. This level of multiplication is not seen with other families of insect viruses that have more restricted tissue multiplication and thus lower productivity per insect. In North America and the EU, such systems were developed during the 1970s and 1980s, notably for *Helicoverpa (Heliothis) zea* nucleopolyhedrovirus (NPV) (Ignoffo, 1973) and *Lymantria dispar* NPV (Shapiro et al., 1981) and the generic issues of industrial production were identified (Shapiro, 1986; Shieh, 1989). These basically simple in vivo systems for BV have been adopted in South America, Asia and South East Asia for producing a range of BV, mostly NPV but also some granuloviruses (GVs); however, production in Africa has only begun within the last few years. In India, baculoviruses particularly the NPVs of *H. armigera* and *S. litura* have been reported to be effective on different crops and commercial-scale production of NPVs was begun in the late 1990s and has expanded since (Rabindra et al., 2003; Srinivasan et al., 2019; Kumar et al., 2019).

Detailed descriptions of commercial production systems for BV are scarce. A few have been produced by public sector researchers (Shapiro et al., 1981; Grzywacz et al., 2004; Moscardi et al., 2011) as generic training material for small scale commercial producers but only one by a commercial producer (van Beek and Davies, 2009). A comprehensive source of technical advice on the production and handling of baculoviruses was compiled in Hunter-Fujita et al. (1998). The BV production system consists of several separate component stages, insect rearing, insect infection and virus multiplication, insect harvest, processing and formulation. A key element to successful systems is the quality control needed at all stages to ensure that production is continuous and efficient (Jenkins and Gryzwacz, 2000). While the system is basically simple in concept, a high level of competence and close attention to protocols is essential if a quality product is to be produced cost-effectively.

The quality and quantity of host larvae, a dose of inoculum and postinoculation incubation conditions used for BV production are major factors in in vivo production. A number of studies have established that the optimum age/weight of larvae is crucial in production efficacy for individual species (Cherry et al., 1997; Grzywacz et al., 1997; Senthil Kumar et al., 2005; Subramanian et al., 2006; Biji et al., 2006). Recent studies also highlight the influence of postmortem incubation on baculovirus yields and contaminants (Ramírez-Arias et al., 2019). If larvae are inoculated when either too small or too large, the production of occlusion bodies is reduced. Mass production is thus optimized if there is a controlled supply of larvae available at a specific standard age/weight. To achieve this consistently, most commercial operations utilize a supply from custom-built larval production plants that ensure an adequate supply of healthy larvae. Attempts to utilize insects collected from the wild have been tried (Ranga Rao and Meher, 2004; Grzywacz et al., 2014); although this is a potentially cheap solution, it can compromise production by inadvertently including other insect species, in which the BV will not replicate. It may also introduce contamination by other insect pathogens whose presence may interfere with successful BV replication. The product from such wild-collected insects is not recommended unless the insect supply is adequately monitored and selected.

The rearing of large numbers of healthy standardized larvae over a long period is a challenge, as such cultures are vulnerable to infection by other lethal parasites or pathogens and consequently the culture can collapse and halt virus production. Culture lines can also be contaminated by parasites or pathogens, which have a more subtle impact, causing gradual loss of vitality and a decline in target BV production. The systemic spread in cultures of parasites such as *Nosema* and *Variamorpha* spp. are often the cause of gradual colony decline in some small-scale BV production laboratories (Grzywacz et al., 2004). This also can have a serious impact on BV purity and productivity, as harvested insects come to contain increasing levels of the contaminant and reduced levels of the target BV. To ensure a healthy culture for production, it is essential to initiate development from clean insect lines and to rigorously apply adequate sanitation measures to exclude contamination once a clean culture is established. The selection and selective rearing of clean culture lines is an important task that can be very time-consuming. Insects from the wild are commonly infected with a

variety of pathogens and parasites and it may take many months of careful rearing to eliminate these by selective breeding (Grzywacz et al., 2004). In starting new production systems accessing already established clean cultures is recommended and can save a great deal of time and effort. To guard against catastrophic culture contamination, rearing the insects at a physically separate facility to the main virus production facility is often adopted. Establishing separate backup insect cultures with research partners or collaborators are also strongly advised as insurance against problems with the main colony.

The provision of a hygienic, cost-effective insect diet is important for many small scales or low technology producers. The use of a diet from grown plant material may be needed by some species not adapted to artificial diets but such material needs careful monitoring as it could bring in pesticide residues or pathogens dangerous to the health of the insect production culture. In India, some use is made of soaked legumes such as chickpea as a food source for insects such as *H. armigera* (Ranga Rao and Meher, 2004). The preferred option for insect rearing is an artificial diet made from standardized ingredients and details of diets for various species are available (Singh, 1977; Singh and Moore, 1985; Anderson and Leppla, 1993), preferably heat sterilized. Diet cooking protocols that involve autoclaving have the advantage of effectively sterilizing as well as cooking insect diets. Diet production costs are a major item in in vivo BV production, and a very significant part of this can be the gelling agents, multivitamins and mineral mix used in standard diets. Agar is the agent of choice in research cultures, but this is expensive, and many producers have developed cheaper solutions using gelling agents from local food industries. Similarly, cheaper multivitamins available in the pharmaceutical sector can replace high-ended scientific-grade multivitamin products.

An important issue is the quality of the virus inoculum. The use of highly purified inoculum of the selected strain is one of the most important measures contributing to trouble-free virus production. Inoculum should ideally be purified by established centrifugation protocols (Hunter-Fujita et al., 1998) and the genetic identity checked using restriction endonuclease or polymerase chain reaction analysis. This is not only to ensure that the selected strain alone is produced but that other potentially contaminating viruses are excluded. Some BV species such as the GV of *S. littoralis* can, when in a mixed inoculum, infiltrate cultures and outcompete the faster killing NPV, thus contaminating the product and lowering its performance (Hunter−Fujita et al., 1997). The recycling of product as inoculum without purification and strain verification should be avoided as this can result in the buildup of contaminating parasites and pathogens; it is a flawed approach that has probably done the most to degrade the BV product quality of small producers in the past (Kennedy et al., 1999). Once again selected strains should be lodged in separate culture collection facilities both as a product reference and as a backup in case of accidents at the main facility.

Effective multiplication of viruses in vivo is usually dependent upon the provision and maintenance of appropriate rearing conditions for the host. Temperature and humidity need to be kept within defined limits to avoid stressing infected insects such that they die before virus replication is completed (Subramanian et al., 2006). While some small-scale producers rely on ambient conditions in rearing facilities, this can have very deleterious consequences for product quality especially during periods of high humidity. One interesting development to increase productivity is the use of juvenile hormones in the insect diet to improve the insect's growth and BV productivity (Lasa et al., 2007). Inoculated insects need to be reared in containers during the virus multiplication period. In production in northern countries, disposable container handling systems are common to facilitate good hygiene. In LIC, low-cost producers often opt for reusable containers which require effective cleansing procedures if contamination is to be avoided. Some insect species such as *Cydia pomonella* can be reared communally during virus multiplication, but some of the most important hosts such as *Heliothis*/*Helicoverpa* species and *Spodoptera* spp. are cannibalistic in later instars; so, these must be reared individually to avoid excessive loss of virus. Compartmentalized trays are generally used to rear such insects and a number of different designs have been developed and adopted (Fig. 15.3), which are then incubated in stacks (Fig. 15.4), though detailed descriptions are sparse (Grzywacz et al., 2004; van Beek and Davies, 2009). As often is the case in BV production, details of these systems and their relative merits are usually not publicly available, as producers rightly perceive these to be crucial commercial intellectual property that underpins market advantage.

Infected larvae are harvested once BV multiplication is complete; in most tropical pest species 5−7 days is sufficient to reach optimal BV production (Cherry et al., 1997; Gupta et al., 2007; Van Beek and Davies, 2009). The harvesting of the larvae is commonly accomplished by whole containers on a preselected day, though in some small production units optimally infected insects are harvested individually over 2−3 days. Harvesting insects at or near death can maximize virus occlusion body production while limiting the microbial contamination load (Grzywacz et al., 1997). While there is little evidence that such contaminants pose a significant risk (Podgwaite et al., 1983), registered virus products usually have maximum allowable levels of bacterial contamination that must be adhered to. Currently, the international norm is the OECD issue paper on microbial contamination limits (Anonymous, 2011), but individual countries sometimes have more stringent standards, as is the case in South Africa. In virus-infected insects, after death,

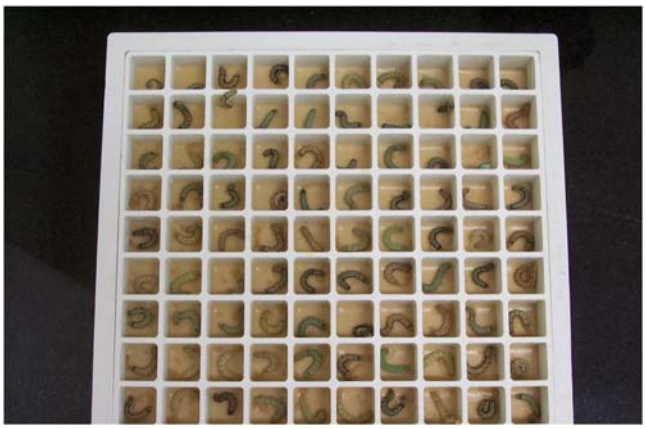

FIGURE 15.3 Compartmentalized tray used for rearing baculovirus infected insects.

FIGURE 15.4 Compartmentalized rearing trays being incubated in stacks to mass propagate Baculovirus.

saprophytic bacteria multiply quickly so harvesting delays increase the load of contamination (Lasa et al., 2008). Harvesting of infected insects can be done with suction devices or automated harvesting systems but individual hand collecting of infected insects is also common but labor intensive. In some systems dead insects, diet, and feces are all harvested and processed together (Grzywacz and Moore, 2017). This decision may depend on whether larvae can be harvested intact or if they rupture before harvesting due to the expression of viral chitinase and/or cathepsin genes (Hawtin et al., 1997). It also depends on whether the larvae are surface feeders or cryptic (within the diet) feeders. Even though symptomatically infected cryptic feeding larvae tend to move out of the diet, lying on top of the diet or hanging from the sides or lids of virus incubation containers, there is often an unacceptably high percentage of infected larvae that remain within the diet (Moore, 2002b) and would be lost if the entire diet was not harvested. Such a practice may exacerbate product contamination issues.

Once harvested, the cadavers and viruses are frozen and stored prior to processing and formulation. Exact details of specific processing and formulation systems are sparse from commercial sources, though information from public sector producers is more available (Grzywacz et al., 2004: Van Beek and Davies, 2009). Larger insect parts such as skin, jaws etc. are generally removed during processing by filtering, to avoid blocking spray apparatus; but other insect debris is almost always left in formulations since it is known to improve the UV stability of BV products (Burges and Jones, 1998). Some BV systems include a primary centrifugation step to further remove gross insect debris prior to the

preparation of BV for formulation. The subsequent formulation of BV varies between producers; a generic discussion of formulation issues is presented in Burges and Jones (1998) and Grzywacz and Moore (2017) and can also be found in Behle and Birthisel (Chapter 14 of this volume). Details of formulations used by producers are generally kept secret for commercial reasons. The lack of formulation technology know-how is emerging as a technological constraint in LICs that needs to be addressed through specific research or knowledge sharing with private or public sector formulation researchers. Early BV formulations were simple aqueous suspensions of homogenized filtered insects. These aqueous suspensions needed to be stored frozen and distributed in cool chains, which was a significant barrier to commercial uptake in LIC. To improve storage and handling characteristics, some production is as wettable powders formulated with clays or talc, or with more stable liquid suspensions often incorporating glycerol as an antibacterial carrier (Subramanian et al., 2007; Lasa et al., 2008). Crude suspensions of NPV need drying to prevent loss of activity or adverse effects on storage characteristics; air drying, freeze-drying and spray drying are all possible (Tamez-Guerra et al., 2002; Grzywacz et al., 2004). These formulations can extend active shelf life at ambient conditions and generally benefit from freezing/refrigeration for long term storage; they are also more amenable to short-term ambient storage prior to use by farmers. In liquid formulation, baculovirus products must be refrigerated, which can be a problem in both the distribution chain and by the end-user in LIC. However, refrigeration of dried formulations is also preferable (Behle et al., 2003). Consequently, it remains a challenge to produce BV formulations that could match the 2 years of stability at ambient temperature that remains the benchmark for chemical pesticide stability. River Bioscience, in South Africa, sells their liquid formulation baculovirus products with a 30-month shelf life, albeit conditional on refrigeration. However, as most of their end-users are large commercial farmers, cold distribution and storage are possibly less problematic than elsewhere in LIC.

Successful BV production needs an effective quality control system but there is no single method for quickly and accurately determining BV activity. The physical numbers of virus occlusion bodies or OB (Fig. 15.5) can be counted by standardized microscopical methods (Evans and Shapiro, 1997), but activity can only be estimated through insect bioassays. Many standardized bioassay methods for insects exist (Evans and Shapiro, 1997; Jones, 2000), but establishing and maintaining a bioassay program is demanding. However, it is only by incorporating real-time counting of OB and bioassays, that production quality can be continuously monitored and failings or problems in production detected and resolved. Inconsistent quality and failure to achieve standard levels of product quality have been among the most important problems in scaling up and maintaining baculovirus production in both public and private sector programs (Grzywacz and Moore, 2017). This is particularly a problem in LIC, where facilities and resources may not be optimal. One solution for smaller producers is to link up with research institutes to carry out routine quality control operations like a system used in Brazil where EMBRAPA operates a quality control laboratory for a number of production companies (Moscardi, 2007). This would also include regular testing of baculovirus samples for levels of bacterial contamination and occasional testing for human pathogens, such as *Shigella* spp., *Salmonella* spp., *Vibrio* spp., and *Escherichia coli* (Grzywacz and Moore, 2017), despite there being no reports to date of such pathogens in baculovirus samples (Jenkins and Gryzwacz, 2003).

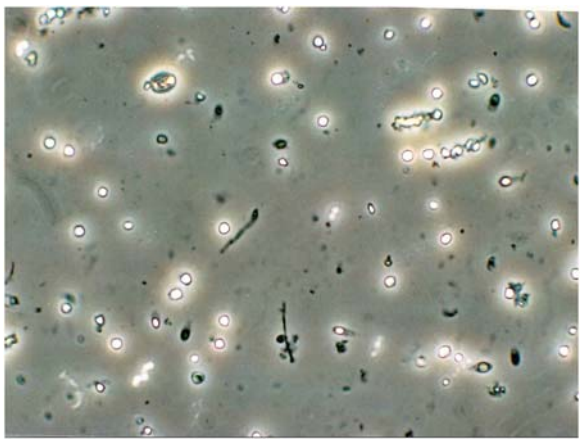

FIGURE 15.5 Infective particles or occlusion bodies (OB) of nucleopolyhedrovirus under ×400 phase contrast microscopy. OB are seen as bright refractive crystals.

15.8.1 Country case studies

15.8.1.1 China

While China might have been classified as a developing country at the end of the last century today it is clearly not so currently; but it seems possible it is the greatest user and producer of BV worldwide (Yang et al., 2012) and is thus an interesting case study in BV scaling up. The situation of BV production in China was reviewed by Entwistle (1998) when BV production was recently established. BV production has since expanded significantly with at least 12 EP virus species registered (Sun and Peng, 2007; Yang et al., 2012) and production has been estimated to be 500 metric tonnes of product, though most of this is *H. armigera* nucleopolyhedrovirus (HearNPV) products (Xiulian Sun, Personal Communication); but other authors have estimated the total of product material may have reached 2000 MT (Wang and Li, 2010; Yang et al., 2012). However, details of production systems, quality and regulation which would certainly prove extremely interesting are poorly reported in non-Chinese literature. One unusual feature is the production of combination Bt and BV products in China; these include combinations of GV + Bt against *Pieris rapae*, NPV + Bt against *S. litura*, and NPV + Bt against *Biston suppressaria* (CPL Business Consultants, 2010). The effectiveness of these combination of products is unclear and their sustainability and genuine utility in IPM or IRM packages remain to be demonstrated.

15.8.1.2 India

There has been a considerable body of research into microbial pesticides in India and a wide range of EP have been investigated in a variety of cropping systems and local production of these agents developed dating from the 1960s (Koul et al., 2003; Mishra et al., 2020). There are 141 private sector laboratories involved in biopesticide production (Mishra et al., 2020), with at least 32 commercial companies active in biopesticide production, with additional IPM centers under the Ministry of Agriculture and the Department of Biotechnology strongly supported efforts to produce selected biocontrol agents (Rabindra and Grzywacz, 2010; Mishra et al., 2020). Though, there has been strong policy support for expanding IPM and the associated use of EP products, to date biopesticides constitute only 3% of the pesticides market and of this only 4% are viruses (Tripathi et al., 2020). In India, many potential BV were identified and studied; but the usefulness of only a few has been demonstrated on a field scale. These include the NPVs of *H. armigera*, *S. litura*, *Amsacta albistriga*, the teak defoliator *Hyblea puera*, and the GV virus of the sugar cane borer (*Chilo infuscatellus*) and diamond back moth *P. xylostella* (Venakumar et al., 2005; Subramanian et al., 2010; Lacey et al., 2015; Kumar et al., 2019). The NPV of *H. armigera* is the most widely studied followed by that of *S. litura*, due to their nationwide pest status on economically important crops like cotton, pulses and oilseeds.

A few important local biopesticide producers dominate the commercial production BV, though many other producers exist (Mishra et al., 2020), the most common registered baculoviruses are *H. armigera* NPV products with 22 registered and *S. litura* NPV with five registered (Kumar et al., 2019), However, the majority of biopesticide production in India, up to 70%, is through the public sector organizations with various government research institutes, state biocontrol laboratories, state agricultural universities and some farmer extension organizations prominently (Mishra et al., 2020). During the early period of development, the virus was produced from field-collected larvae; but subsequently, EPs were brought under the purview of the insecticide act, necessitating registration with the Central Insecticides Board (CIB); thus, the emphasis shifted toward production in larvae mass-produced in semisynthetic diet to address the quality issues (Rabindra et al., 2003). The registration system for EP has been favorable and flexible allowing provisional registration of approved agents before full dossiers are submitted, which has facilitated production and registration by small and medium enterprise producers (Kulshrestha, 2004; Rabindra and Grzywacz, 2010). This success in promoting biopesticides can be seen in that at least 15 microbial agents have been developed into registered products with 970 different formulated products registered, though this also includes many locally produced botanical pesticides (Kumar et al., 2019). The marketing of baculoviruses was initially largely through the government subsidy programs and to date there are only limited direct sales to the farmers. Due to erratic pest occurrence, the demand has not been predictable and some of the companies that registered their NPVs have since ceased to produce the virus and at present uptake is limited. Much of the production of *H. armigera* NPV and *S. litura* NPV is by medium-sized commercial units or small-scale cottage industries (Kumar et al., 2019).

Quality standards for products are established, but the regulation and enforcement have in the past been rather poor and have affected consumer confidence (Kennedy et al., 1999; Alam, 2000). This issue of lack of production experience and poor-quality control is still one that continues to plague Indian biopesticide products (Mishra et al., 2020). Surveys to test the quality of biopesticides are conducted, and while some manufacturers clearly meet accepted standards

(Kambrekar et al., 2007; Srinivasa et al., 2008), other reports indicate significant quality concerns, especially from new and inexperienced producers, as well as the existence of fake microbial products (Kumar et al., 2019). A system of referral laboratories accredited by the Indian Department of Biotechnology for quality testing has been established, but enforcement of standards has in the past been uncertain and remains a serious concern (Shetty et al., 2008; Mishra et al., 2020). It is also reported that delays in registration and the registration cost are factors contributing to the sale of unregistered and poor quality biopesticides (Kumar et al., 2019).

15.8.1.3 Thailand

Biopesticides production in Thailand began in the late 1980s when researchers produced and studied BV and Bt to control insecticide-resistant pests such as *H. armigera* and *Spodoptera exigua* (Jones et al., 1998). Field research demonstrated the efficacy of BV for controlling key pests (Kolodny-Hirsch et al., 1997). Subsequently production facilities for *H. armigera* NPV, *S. exigua* NPV and *S. litura* NPV were set up by the Department of Agriculture in 1995 at Kasetsart University (Jones et al., 1998; Skovmand, 2007), with capacity of around 1000 L BV product per annum (Ratanasatien et al., 2005). In 2004, the Department of Agriculture collaborated with the National Science and Technology Development-Agency (NSTDA) to set up a BV pilot plant. The BV pilot plant was established in 2007 in the area of Thailand Science Park Bangkok. The new pilot plant can expand the production capacity of BV product up to 3000 L/annum. Since 2008, the BV pilot plant has also produced BV for the control of tea leaf rollers (*Homona magnamina* and *Adoxophyes orana*) under the collaboration with Arysta Life Science Corporation, Japan.

15.8.1.4 South Africa

Research into biopesticides and insect viruses is well established in South Africa dating back to pioneering work by L. L.J. Ossowaski on the control of bagworm (*Kotochalia junodi*) in the 1950s and V.H. Whitlock on *H. armigera* NPV in the 1970s (Kunjeku et al., 1998). A strong entomological research base and the high economic value of the horticultural export sector were undoubtedly important factors that have contributed to the establishment of biopesticide production in South Africa. A major focus was the control of false codling moth (*Thaumatotibia (Cryptophlebia) leucotreta*), a pest refractory to control by chemicals. Research on the potential of the *T. leucotreta* granulovirus (CrleGV) as a control agent was initiated (Moore, 2002a; Moore et al., 2011), which was followed by the establishment of local in vivo production of CrleGV and its subsequent registration by River Bioscience (Moore et al., 2004), currently the only commercial baculovirus product manufacturer in South Africa. Production of this virus is now well established with facilities and systems for mass rearing virus-infected insects (Fig. 15.6A and B) and processing the harvested virus

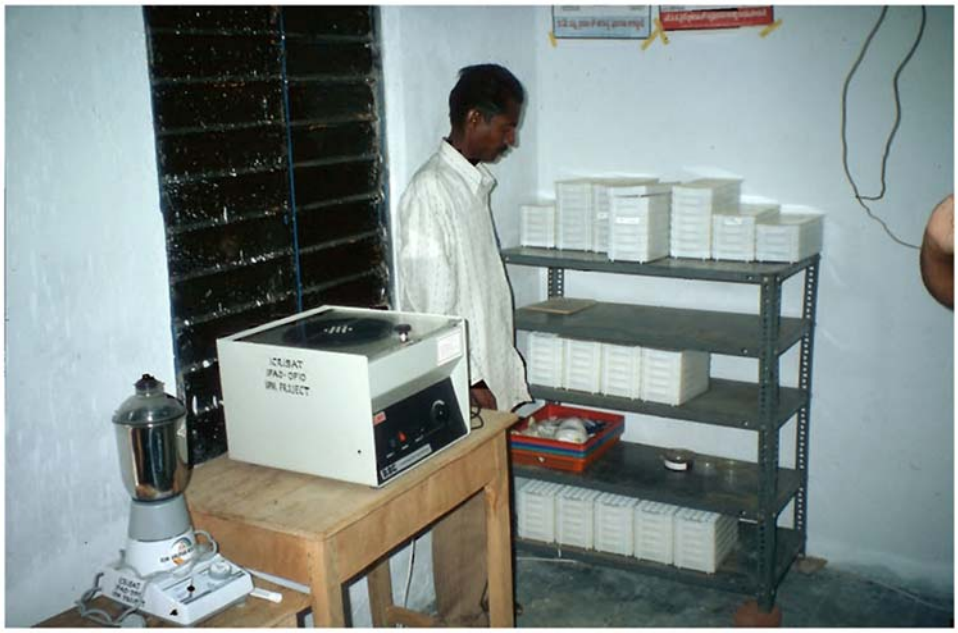

FIGURE 15.6 Village biopesticide production unit in India for production of NPV.

FIGURE 15.7 (A) Insect rearing trays for mass propagating baculovirus. (B) Baculovirus infected insects in rearing trays.

(Fig. 15.7) and has been used in the field for more than 15 years, becoming a mainstay of the *T. leucotreta* control program (Moore, 2021). Further research into the efficacy of the virus and its improvement is continually underway (Moore and Jukes, 2020; Moore, 2021), including selection for UV-resistance and synergism with other EP. River Bioscience has also initiated the production of a novel baculovirus, the *Cryptophlebia peltastica* nucleopolyhedrovirus (CrpeNPV) (Moore, 2021), which although isolated from the litchi moth, *C. peltastica*, is also virulent against *T. leucotreta* and *Cydia pomonella*, the codling moth (Marsberg et al., 2018).

15.8.1.5 East Africa (Kenya and Tanzania)

In East Africa, both Kenya and Tanzania have been centers of research into BV for pest control with efforts in Kenya focused on diamond back moth (*P. xylostella*) and other lepidopteran pests of the horticultural industry (Wabule et al., 2004) and in Tanzania on the use of endemic BV for control of African armyworm *Spodoptera exempta* (Walker) (Grzywacz et al., 2008). Production of *P. xylostella* GV and HearNPV is underway in Kenya and some details of the production systems being used by Kenya Biologics are published (Van Beek and Davies, 2009). Both BVs are now registered in one or more countries. A facility created for *S. exempta* NPV was built in Tanzania in 2008−11. The Tanzanian production system used field production of the virus, using a modification of the EMBRAPA AgNPV (*A. gemmatalis* NPV) system; this is deemed technically feasible because of the large synchronous outbreaks of larvae at high densities which are a feature of this pest (Mushobozi et al., 2005; Grzywacz et al., 2008). The facility never became fully operational, due in part to a subsequent decline in *S. exempta*'s status as a pest and as the focus of pest control shifted to coping with the arrival of the invasive fall armyworm (*S. frugiperda*) (Day et al., 2017), which was not susceptible to *S. exempta* NPV. It remains to be determined if such field systems are economically viable or can meet the requirement for large amounts of viruses that will be needed when periodic major outbreaks occur as these can cover hundreds of thousands of hectares in sub-Saharan Africa.

15.8.1.6 South America and potato tuber moth

Production of the GV of *Phthorimaea operculella* (potato tuber moth) for control of this pest in potato stores is well established in South America through a series of production centers in Peru, Bolivia, Ecuador and Colombia (Winters and Fano, 1997; Sporleder and Kroschal, 2008). The larvae used for virus production are reared on potatoes and then placed on fresh potatoes surface inoculated with GV, where they are allowed to grow for 20−30 days for the infection to develop and the GV to multiply. The dead larvae are harvested, then macerated and mixed with a clay or talc carrier which is then air-dried and powdered to produce a formulation. Production is carried out at the International Potato Center in Peru, at PROINPA in Bolivia and by various commercial producers and sold under a variety of trade names. This product is mainly targeted at protecting seed potatoes in the nonrefrigerated stores of small-scale farmers. Thus, total production is presently limited but expansion to larger-scale producers was planned (Lacey et al., 2010) but the widespread appearance of new potato moths, *Symmetrischema tangolias*, and more recently, *Tecia solanivora*, neither susceptible to the original virus isolate undermined the sustainability of this product. One of the newer versions of the

product Matapol, produced in Bolivia, is a co-formulation between Bt and *P. opercullela* GV to give control of *S. tangolias* as well as the *P. operculella* (Kroschel et al., 2020). Subsequently, research identified that mixtures of *P. operculella* GV strains could be used to overcome the problem of controlling *T. solanivora* (Espinel-Correa et al., 2012) and products with action against multiple potato moths are now in production (Haase et al., 2015).

Production of other BV is established in several South American countries (Table 15.1); however, published details of production are lacking so that detailed discussion of the production is not possible (Olivera, 1998; Moscardi, 1999; Sosa-Gómez et al., 2008; Sosa-Gómez et al., 2020). One interesting development is the production by the Agricola el Sol company of Guatemala of a BV product containing both the NPV of *Autographa californica* and the NPV of *S. albula* (W.). Besides this, *S.* NPV is produced in Guatemala, *Erinnyis ello* GV is produced in Brazil and Colombia and *H. zea* NPV and *H. armigera* NPV are produced in Brazil (Haase et al., 2015).

15.9 Other production systems

Most BV biopesticide products are produced through infecting and rearing insects from laboratory cultures. However, with some pests, the concept of infecting and producing the BV from insects in the wild is feasible for mass production. Many host species are seemingly not suitable for this approach, but it has been commercially viable for a few. Insects that have larvae appearing at a high density, and for which generations are highly synchronized and visible, are probably the most suitable for this system. The system may also be adopted for species where either synthetic rearing diet has not been developed or is too expensive for commercial mass production.

The largest and best-documented field production system is that developed in Brazil by EMBRAPA for *A. gemmatalis* NPV, in a program that produced enough BV to treat 2 million ha of crops (Moscardi, 1999). The system involves scouting for natural pest infestations in farmer's fields where high concentrations of the target pest are identified at a suitable stage; and then, these insects are used for production. At an appropriate larval stage, these chosen pest outbreaks are sprayed with AgNPV, the plots inspected daily, and when peak appearance of the virus killed larvae occurs, pickers are sent to harvest infected larvae. Peak harvest is 8−10 days postinoculation and pickers are paid per volume of larvae collected. Production areas may require 200−300 pickers and may yield up to 600 kg of larvae per location (Moscardi, 2007). The franchising of this system, by EMBRAPA to a number of private producers, the largest being Codetec, underpinned an expansion of AgNPV production that involved the collection of up to 20 tons of caterpillars. Infected larvae were formulated with a kaolin carrier and air-dried to produce enough formulated product to apply to 2 million ha per annum. EMBRAPA remained central to production acting as a quality control center for the production companies. Unfortunately, there has been a dramatic demise in the extent of the program. Reduced demand for AgNPV resulted from the advent of no-till agricultural systems in Brazil, resulting in soybean growers applying herbicides before sowing. Consequently, many farmers acquired agrochemical packages from suppliers, including insecticides, reducing the need for AgNPV. Recently this virus was reported to be applied to only about 200,000 ha/ year, one-10th of its previous use (Haase et al., 2015; Sosa-Gómez et al., 2020).

The use of insects collected from the field is also part of an interesting initiative developed in India. To meet the challenge of producing low-cost biopesticides for use by the poorest farmers, a system of "IPM villages" was developed. *H. armigera* NPV had been shown to be effective in controlling *H. armigera* on a number of crops in India (Rabindra et al., 1992; Visalakshmi et al., 2005) but the high cost of commercially produced NPV discouraged its use by poor farmers. To overcome this, village-level production of HearNPV was established as part of a program of IPM promotion in 2005−07. This involved collecting *H. armigera* by shaking infested pigeon pea plants and collecting the dislodged larvae. The larvae were then taken to the village where a local NPV multiplication unit had been established (Ranga Rao and Meher, 2004). The cost of the simple equipment needed was about US$500 and consisted of larval rearing containers, a homogenizer, a simple centrifuge and the facilities themselves, which were shared by participating farmers (Fig. 15.8). During the program, 96 village NPV production units were established in India and Nepal (Ranga Rao et al., 2007). However, despite this progress, it has been suggested that such farmer production has issues ensuring the financial sustainability of these initiatives in the absence of outside support (Tripp and Ali, 2002). A simple field production technique was also adopted for propagating the NPV of the pea nut hairy caterpillar, *A. albistriga*. The virus was applied to the crop heavily infested by the larvae after which the diseased larvae were collected and processed (Veenakumari et al., 2007). This use of the NPV by the local community and self-help groups avoids the necessity for registration, but so far has not spread further for want of follow up programs. Another such program was followed in Zimbabwe against the semilooper complex attacking soybean. Farmers were advised to collect infected larvae in the field, at the point of death, and relocate them in soy fields in patches where semilooper infestation was observed to be

FIGURE 15.8 Modern baculovirus processing equipment, homogeniser, filter and bulk temperature-controlled storage of baculovirus suspension.

the heaviest. Although not much is known about the virus, it is effective in controlling all four species of semilooper in the complex, and thus may consist of more than one species of virus. In high rainfall areas, most farmers report natural virus epizootics every year and have no need for artificial infection (Kunjeku et al., 1998).

15.10 Generic production issues

15.10.1 Product quality

The quality of biopesticide products in LIC can be extremely variable and this has been a significant issue impairing the uptake of biopesticides in some countries (Alam, 2000). From a survey of three *Metarhizium*, two *Beauveria* and one *Trichoderma* products (two from China, two from Indonesia, one from India, and one from Colombia), only one product, a *Beauveria* sp., showed low levels of contamination and a high germination level (Jenkins and Gryzwacz, 2000). Surveys of BV products have also shown products lacking of active ingredient and highly contaminated with bacteria and other microbial contaminants (Kennedy et al., 1999). Objective published data on this issue is sparse and it is probable that this is a seriously underreported issue in many LIC countries. The situation can be exacerbated where biopesticides are being sold unregistered and therefore unregulated. Additionally, dealers may market cheap illegal imports because legitimate local supplies are insufficient, or because local product registration systems are too slow or uncertain to facilitate registration causing users to obtain essential inputs illegally. South Africa is seeking to overcome this very real problem, which can tarnish the name of the biocontrol industry as a whole, by the formation of the South African Bioproducts Organization (SABO); which both, self-polices the industry and lobbies for the cooperation of the regulator (Hatting et al., 2019). Large-scale use of biopesticides began in Brazil and China in the 1970s (Li et al., 2010); but many small companies disappeared in the 1980s, due to the poor quality of the product in Brazil and reduced government support in China. Interestingly, neither Brazil nor China published much on mass production systems which may be a partial explanation for the poor quality.

In some countries, a problem has been in production systems, which have been established or rapidly expanded without sufficient attention to quality control issues. Small producers may set up without in-house capacity to monitor active ingredients or microbial contamination. Improved product safety greatly reduces losses resulting from the contaminated product being rejected (as well as leading to a failure to supply a product), increasing both financial return and user confidence (Jenkins and Gryzwacz, 2003). Such producers could overcome this by linking with local research institutes, such as is the case in Brazil with the EMBRAPA system for AgNPV production. In Kenya, a partnership between the Real IPM company and the International Center of Insect Physiology and Ecology formed the basis for the development of local *M. anisopliae*-based products (Akutse et al., 2020), while in India companies have also formed relationships with Universities, National and International Research Institutes to enhance technical development of new products and develop better quality control (Rabindra and Grzywacz, 2010; Kumar et al., 2019). Several papers detailing acceptable quality standards have also been published (Jenkins and Gryzwacz, 2000, 2003) and provide essential guides for quality control of fungal and viral biocontrol agents.

15.10.2 Scale of production and application rates

It is often underestimated how much fungus may be required for pest control and variable conidial yields can greatly alter substrate volumes required (Table 15.2). The 25.9 million ha treated in the African locust plague of 1986–89 would have required 1300 tons of conidia (produced from perhaps 30,000 tons of rice). Posada-Flórez (2008) working with *B. bassiana* against the coffee berry borer, *Hypothenemus hampei* in Colombia, similarly calculated that application to 1000 ha would require 92.3 tons of rice to produce the spores. Further examples are given by Bartlett and Jaronski (1988) who calculated conidial numbers required at standard field rates of 1×10^{13}/ha; extrapolating, the treatment of 4–5 million hectares of, in their example, Illinois corn would require 500–1000 tons of *B. bassiana* conidia. Even where mycoinsecticides have been used on what is considered a large area, the figures reveal a massive scale-up is required. China used *B. bassiana* on up to 1.3 million ha until the 1980s and uses probably half that figure now (Li et al., 2010); this represents 13,000 km^2 out of the 1.4 million km^2 of arable land (with a further 1.8 million km^2 of forestry) in China. More intensive production is required and Li et al. (2010) report that new private companies equipped with modern large liquid and solid fermenters or other fermentation facilities have begun producing mycoinsecticides in China in recent years. Where scale-up requirements are substantial, the use of low technology processes is likely to fail. According to Roberts and St Leger (2004) "Difficulties in

TABLE 15.2 Examples of conidial yields in a range of low-intensity production systems.

Fungus	Substrate	Yield/g	References	Notes
M. anisopliae	Rice bran/husk	2.3 × 10*10	Dorta et al. (1996)	Considered 80% of maximum theoretical yield
M. anisopliae	Rice	2.5 × 10*9	Barajas et al. (2010)	
M. anisopliae	Rice	5.3 × 10*10	Prakash et al. (2008)	Yields of 4.6 and 4.2 × 10*10 for sorghum and barley respectively
M. anisopliae	Rice	2.7 × 10*9	Chen et al. (2009)	
Metarhzium flavoviride	Rice	2.2 × 10*9	Chen et al. (2009)	Characterized as *M. anisopliae* var *acridum* by Fernandes et al. (2009). (*M. acridum*)
M. acridum	Rice	1.5 × 10*9	Jenkins et al. (1998)	Small scale mass production of 200kg/year
B. bassiana	Rice	1.8–2.7 × 10*9	Ye et al. (2006)	
B. bassiana	Sorghum	4.2 × 10*9	Rajanikanth et al. (2010)	Six isolates tested, ranging from 2.8–4.2 × 10*9
B. bassiana	Rice + wheat bran	2.9 × 10*10	Dhar and Kaur (2011)	At 35% moisture and 1.5% yeast extract in liquid nutrient.
B. bassiana	Wheat	1.2 × 10*9	Sahayaraj and Namasivayam (2008)*	Range of grains tested, yielding around 1 × 10*9
B. bassiana	Rice	4.6–5.4 × 10*9	Chen et al. (2009)	Two isolates examined
B. bassiana	Rice	2.0 × 10*11	Alves and Pereira (1989)	Harvesting method may have exaggerated yield
B. bassiana	?	2.6 × 10*10	Bradley et al. (1992)	Mycotech pilot production
Nomuraea rileyi	Crushed sorghum	2.8 × 10*9	Devi et al. (2000)	Air exchange required
Isaria fumosorosea	Sorghum	1.0 × 10*10	Sahayaraj and Namasivayam (2008)[a]	Range of grains tested, yielding around 0.8–1.0 × 10*9
Isaria fumosorosea	Rice	1.3–1.8 × 10*9	Chen et al. (2009)	Two isolates tested
Isaria farinosa	Soybean meal or broken corn	1.05–1.32 × 10^9	Mascarin et al. (2010)	

[a] *Sahayaraj and Namasivayam (2008). Inconsistencies in yield recorded between text and table, but these do not alter general patterns.*

producing *M. anisopliae* described in Krassilstchik (1888) are woefully similar to those expounded by DWR (Don Roberts) in Brazil in 2002." Yields, as shown by conidia/g can vary greatly (Table 15.2); but 5×10^{10} conidia/g of the substrate may not be unreasonable by incremental improvements. Some truly innovative approaches may add substantially to this, at which point the economics begin to favor biopesticides overwhelmingly.

For baculoviruses, field application rates may be in the range of 5×10^{11} OB/ha for certain viruses, for example, AgNPV, but for most NPV and GV field rates lie in the $1-5 \times 10^{12}$ OB/ha range (Copping, 2009; Gwynn, 2014). Even with the relatively low rate used for AgNPV, 40 tons of infected insects were needed to treat 1 million hectares of crop in a season (Moscardi, 2007). Thus, the mass production of very large numbers of insects will be needed if BVs are to meet the pest control needs of major field crops in the future. Currently, it is uncertain that the existing in vivo systems can be scaled up reliably to meet this need - thus the interest in large scale tissue culture production (Granados et al., 2007). On the other hand, the expansion of BV production in China in recent years may have reached 500 tonnes/year (Yang et al., 2012), which shows that larger-scale production is attainable with existing systems. Furthermore, if one considers the mass production capability of a sterile insect facility in terms of virus production capacity, such as that in South Africa for *T. leucotreta* sterile insect releases, it becomes clear how much virus could be produced in one facility of this scale. In 2016, the facility produced and released moths over 18,700 ha at 2000 moths/ha/week (Marec and Vreysen, 2019), equating to a production for use of more than 37 million moths per week. At a production of 1×10^{11} Obs/larva (Moore, 2002a,b) and an application rate of 5×10^{12} Obs/ha (Moore, 2021), the virus (CrleGV) production capacity of the facility would be sufficient for more than 700,000 ha applications. However, such large sterile insect programs and rearing facilities are not commonplace in LIC.

15.10.3 Safety

EP selected for commercial production by virtue of passing a registration system should be inherently safe for use as specified, both as formulations and active ingredients. Therefore, the main safety issues concern hazards in the production of the BCA rather than their use. Generally, the genera of entomopathogenic fungi used in mycoinsecticides are known to be very safe (Zimmermann, 2007a,b, 2008). It is possible to obtain adverse reactions from isolates, but under circumstances that would not occur in field use; Goettel and Jaronski (1997) described serious responses to pulmonary (intranasal) tests of a *M. anisopliae* isolate from Madagascar. In reality, safety is an issue at production, during formulation and use, and for registration. Conidial powder becomes airborne very easily, presenting a respiratory risk; an issue of long standing in fungal production (Rorer, 1913). Today this can be controlled through appropriate safety measures. Chinese production units developed vacuum extracting equipment to harvest conidia, reducing allergy problems resulting from inhalation in the 1980s (Li et al., 2010). Subsequent to production, farmers could be exposed to respiratory hazards from product formulated as powder. Different formulation types can create or reduce the hazard. In Brazil in the 1970s and 1980s, *M. anisopliae* was washed from the rice substrate with water for use against the sugarcane froghopper *M. posticata* (Mendonça, 1992). The liquid contained spores, mycelium, rice fragments, starch and bacteria which rapidly became a significant contamination problem if the product was not applied rapidly after harvest. The problems of contamination developing and of respiratory hazard of powders can be removed by formulating in oils. The LUBILOSA project used an oil suspensible flowable concentrate, essentially a very concentrated spore sludge, which could be diluted easily with oils to achieve the correct concentration for application.

BV has been characterized as inherently safe for use in pest control (OECD, 2002), so safety issues here concern production and incidental microbial contamination. As BVs are produced in insects there are issues around the microbial flora that occurs in live insects and proliferates in dead insects. Several studies have characterized these (Podgwaite et al., 1983; Grzywacz et al., 1997; Lasa et al., 2008). To date none of the studies has identified human or veterinary pathogens, but such microbiological studies are an essential safety measure that should not be neglected. The various systems for low technology production that do not include microbiological screening as a routine part of quality control must remain a cause for real concern. The insects themselves, rather than the BV produced, could also cause health problems, due to potential allergic responses by facility staff to adult wing scales and larval setae.

15.10.4 Economics of production

It is often assumed that a labor-intensive model for mass production of mycoinsecticides is the more appropriate model for developing countries and the capital-intensive model for the wealthy countries. Swanson (1997) examined the economics, based on net present value, of two production systems; the labor-intensive LUBILOSA model and a capital-intensive model based on the Mycotech Corporation. The LUBILOSA production system was better for markets of 20,000 ha/annum, while the capital-intensive model was more profitable at markets of 80,000 ha/annum. The two main

points are that labor-intensive models can be financially viable, but to satisfy potential demand, large production capacity will be required. Most LICs have industries that require a similar level of technology and sophistication (brewing, pharmaceuticals, etc.) and it is likely that any country seriously wishing to use fungal EP will eventually turn to factory systems for the quantities required for broad-acre agriculture. Nonetheless, this may still leave niches for specialized smaller producers. An economic study of Green Muscle use in West Africa suggested that the product was expensive, being produced at US$17/ha at 100 g/ha, compared with the chemical competitor sold in the market at US$12/ha (Groote, 1997). Industrial-scale production would lead to reductions in cost but, more importantly, the effective application rate was later reduced to 50 g/ha with greater experience of using the product. One route for reducing application rates is the development of innovative techniques such as "Lure and infect." Such strategies are also seen as having the potential to enhance the field stability of entomopathogenic fungi (Mfuti et al., 2017). Currently "Lure and infect" products for the management of thrips and fruit flies are being developed by Real IPM in Kenya.

Published studies of the economics of BV production are unfortunately very rare; an exception is a data on the AgNPV field production in Brazil estimating a BV production of $1.28/ha which made it highly competitive with chemical insecticides (Moscardi, 1999, 2007). However, the unusually low application rate for this NPV and the field-based system make it unlikely that this system would be appropriate for many other BV (which need to be produced through mass insect rearing and thus will be much more costly). Generally, to date, BV products have not been produced at a cost less than alternative chemical insecticides. BV production is, for the most part, labor intensive; thus production in low-cost countries such as China, Brazil, India, Kenya and South Africa is seen as a feasible way to reduce product costs. This may be particularly so if production for different purposes is performed in the same facility; for example, production for sterile insect releases or for parasitoid production for augmentation, could be conducted in the summer months, when releases are required. In the winter months, when crops are not bearing, larvae could be used for BV production, due to the refrigerated shelf-life of BV products. Moore (2021) reports that in South Africa the average cost per hectare of a BV spray for *T. leucotreta* is $80, whereas the average cost of a chemical spray is $243. This can be attributed to the fact that the first BV product on the market is locally produced, whereas all chemical insecticides are imported from western countries with stronger currencies. Another driver of EP use is that new generation pesticides, which are increasingly the only effective pesticides against pests resistant to older chemistry, are highly priced and may be costlier than locally produced biopesticides. This should encourage increased production of LIC. Large global biopesticide companies, such as Andermatt (Switzerland) and AgBitech (USA), both specializing in BV, have a large and growing presence in Africa, South America and Asia. Some of their products of relevance to these regions are based on BV such as HearNPV, CpGV and *S. frugiperda* NPV. However, if these products were manufactured locally, rather than Europe and the USA, they would surely become available to farmers at much more competitive prices. *H. armigera* has previously been ranked as the most important lepidopteran pest, at least in southern Africa (Bell and McGeoch, 1996) and the status of the invasive fall armyworm, *S. frugiperda*, has risen dramatically in recent years, in Africa, China, India and other Asian countries (Goergen et al., 2016; Day et al., 2017; Naganna et al., 2020; Sun et al., 2021). Consequently, it should easily be justifiable to produce these BV in affected LIC.

15.11 Requirements for establishing biopesticide industries in less-industrialized countries

15.11.1 Research and information

One common feature of mass production in many LICs is a poor understanding of principles and a lack of awareness of fundamental work. With the internet, there should be less of a problem in obtaining sources of information. While it may be true that some important information is not freely available, there is sufficient accessible material that will allow mass production to be carried to a reasonable level while the isolates are being researched for optimization. However, the need for a certain degree of technical expertise remains. This is important for accurately identifying the pathogen that is being produced, its continued genetic integrity and its purity. This may best be achieved by working with a technical partner with the appropriate expertise and facilities, such as a university of the research institute.

With fungi, a major issue is the need to have a solid substrate for the production of aerial conidia, which is the basis of most production processes (Roberts and St Leger, 2004). Although liquid systems have been developed, the logistics of many supply chains require better shelf life than many liquid systems provide at present. Although blastospores can show improved shelf-life while retained in their original production liquid, there is a belief that these propagules types are not of practical value (Li et al., 2010) despite some evidence for a number of species that good field results can be obtained.

The more exotic propagules such as microsclerotia and submerged conidia (Chapter 11 of this volume) may well have a valuable role in the future, but major advances could be obtained from properly understanding the solid substrate.

There is a belief that the substrate has a vital role in supplying nutrition to a fungal culture, beyond that provided by a liquid nutrient culture, but the substrate's physical characteristics may be more important. Little is known about the physical requirements, and the relationships between surface area and appropriate spaces to optimize conidial production. Relatively poor production from coarse material such as ears of cereals or other husk material may well be due to physical space more than an inadequate carbon: nitrogen ratio. The particle size of cracked rice or millet usually seems to provide good spaces, but increasing the surface area of a substrate may result in disrupting the benefits of the correct particle size. If a solid substrate could be optimized for surface area and air spaces when stacked and made of an easily sterilizable material, so it can be recycled, a number of problems could be resolved.

Certain themes are apparent in promoting improved biopesticide production in LIC (Table 15.3). It is notable that it is in countries where markets are relatively large (such as India, China, and Brazil) or where the export horticulture sector provides an active market for biopesticides and biocontrol agents (for example, Thailand, Kenya, and South Africa) where significant progress in developing an EP industry has been most marked (Wu et al., 2014). Government support was pivotal in establishing most biocontrol programs in countries such as India, Brazil, and China. Funding for research and development of products has been effective where it brought together researchers and industry into productive partnerships. Government/public support is necessary to allow biopesticide businesses to establish. This is clear at research levels, to optimize products, support biopesticide businesses and for creating a commercial environment where biopesticides are viewed positively for their many advantages (Marrone, 2007). In Brazil, India, Thailand and China, national research systems had significant specific biopesticide research programs aimed to generate new knowledge, build local expertise research capacity, and disseminate research findings to Industry (Wahab, 2004). The Indian Council of Agricultural Research, the Department of Biotechnology of the Ministry of Science and Technology support research and development projects for EP products. The focus has been to enhance quality and the Department of Biotechnology is providing funds to the public sector for the generation of toxicological data and has made a provision for sharing the data generated among several potential entrepreneurs so that the cost of registration can be substantially reduced. International donor support has also been important; the LUBILOSA program was funded by a number of donor countries, and the scientific results were published and available and both Brazil and China benefited from this program. The UK Department for International Development, US AID IPM programs and other European donors have funded various biopesticide projects in Asia and Africa (Sweetmore et al., 2006; Skovmand, 2007; Akutse et al., 2020).

Having locally available researchers with biopesticides expertise is a major factor in promoting the development of biopesticide companies. In India, Thailand, Kenya and South Africa the presence of a core of active biopesticide researchers in academia, national research centers or international research centers is playing a key role by promoting biopesticides, influencing policy makers, providing competent contract research services and by training students able to join the industry. In addition, development funding schemes, which enabled northern research expertise to be made available to support local researchers and industry were also of benefit. Research institutes in LIC are often weakest at scale-up and manufacturing, which is essential to turn the research of local biocontrol agents into practical biopesticide products (Harris and Dent, 2000). While there are often nuclei of researchers doing early-stage research on identifying new biocontrol agents and preliminary laboratory and field evaluations, the specific expertise to develop these agents into products can be absent (Grzywacz et al., 2009). This could be overcome with the development of effective establishment of public−private partnerships like those seen in Kenya, India, Brazil, and South Africa.

In addition, existing research funding and peer review reward systems can focus overly on academic research into these early-stage activities. It is felt by many in the biopesticides industry that researchers may have limited experience in working with the private sector. This leads to both sides having inappropriate expectations and reduces the value of collaborations between industry and academics, a situation not confined to LIC alone (Lisansky, 1997). There is a clear need for research funding agencies to build researchers' capacity not only in technical aspects of research but also in enterprise skills so that research can more effectively be translated into practice and into products beneficial to the wider community. Some agencies have specific programs to facilitate academia-industry collaboration and these have been effective in some LIC (Wahab., 2004).

However, existing research is creating improvements. Ye et al. (2006) described the development of a solid-state apparatus consisting of an upright multitray conidiation chamber (and explained how a 200 m^2 factory could produce material for 80,000 ha). Using this apparatus in series, Chen et al. (2009) produced six different isolates of EP simultaneously and achieved yields of some isolates that were three times that of Ye et al. (2006). Optimization of moisture content and yeast extract concentration enabled Dhar and Kaur (2011) to give precise figures for these requirements with different substrates; optimization of pH and moisture content for other substrates could also be accomplished

TABLE 15.3 Features promoting improved mycoinsecticide production, their registration and adoption.

Area	Feature	References
General	Government support essential for most biocontrol programs at initial stages An overview of registration of microbial control agents Biological control status and recommendations for improving update	Li et al. (2010), Gwynn (2016), Barratt et al. (2018), Mishra et al. (2020), East African Community (2019)
Production – mitigation of contamination	Avoid contamination by lowering pH with lactic or acetic acids to suppress bacteria Avoid contamination by lowering pH with 3% lime water to suppress bacteria Avoid contamination by incorporating large amounts of inoculum into the substrate Avoid contamination by incorporating selective fungicides into substrate Increased UV-A tolerance of conidia	Prior (1989), Li et al. (2010), Prior (1989), Prior (1989), Huarte-Bonnet et al. (2020)
Production	Lower costs by use of locally made polypropylene carrier bags which were autoclavable instead of laboratory standard bags Reduce production costs by applying inoculated rice in sugarcane, allowing conidiation to occur in the field Use tap water instead of purified water for increased blastospore production at liquid phase, in Honduras Substrate inoculum in bags done above a pot of steaming water if no laminar flow available Increase production or spores/g substrate by adding silkworm pupae powder to the solid substrate Lower costs by using tray bioreactors Using coffee and cocoa waste, and molasses for culture media Lower costs by using recycled parboiled rice	Jenkins et al. (1998), Bibi Ali (personal communication), Todd Kabaluk (personal communication), Torres et al. (1993), Bai et al. (2020), da Cunha et al. (2019), Kouadio et al. (2019), Barra-Bucarei et al. (2016)
Registration	Common genera such as *Beauveria bassiana* and *Trichoderma* do not need specific safety data in Ecuador In China, many products are sold and used without registration The Pest Management Regulatory Agency (PMRA) does not charge a fee for biopesticide registration and the right to sell in Canada. Also no requirement for residue studies in crops as generally there are no maximum residue limits for biopesticides Qualified presumption of safety may be granted on grounds of taxonomic grouping, intended use and pest knowledge or organism	S. Jaronski (personal communication) Li et al. (2010), Kabaluk et al. (2010), Kabaluk et al. (2010)
Policies	Korean Government is aiming for a 40% reduction (from 2004 levels) in the use of chemical insecticides by 2013 Ministry of Forestry pest management in China required to use biological control measures Guaranteeing minimum crop price might encourage farmers to use nonchemical alternatives may stimulate interest in entomopathogens in Iran Clear national certification systems for organic or chemical pesticide-free crops required in Iran	Kim et al. (2010), Wang and Li (2010), Karimi et al. (2019)
Use	Preconidiated rice can be distributed at the early growth stage, with conidiation occurring naturally in the field (in a humid environment/crop) Application of biopesticides adopting "Lure and infect" strategies	Jenkins et al. (2007), Bibi Ali (personal communication) Mfuti et al. (2017)

(Prakash et al., 2008). The figures can vary greatly among substrates (Table 15.2), for instance, moisture contents of 73% and 22% were optimal for the production of sorghum and rice respectively (Prakash et al., 2008).

15.11.2 Registration and regulation in less-industrialized countries

Commercial companies are clear that registration issues in many countries remain a significant barrier to expanding the role of biopesticides (Ehlers, 2011; Ravensberg, 2011). Products are not produced or registered in many smaller markets if registration requirements are too costly or the processes are uncertain or lack transparency. An issue in many countries has been that registration systems are focussed on the registration of synthetic chemical pesticides and authorities lack the expertise or experience in EP, biocontrol agents and biopesticides. One pragmatic solution, apparently quite common in some countries, is to sell unregistered products. This can all too easily lead to the appearance of spurious, fake and poorly performing products which can quickly result in a reputational loss and kill customer confidence in biopesticides (Alam, 2000; Mishra et al., 2020). This approach while simple is far from ideal; but this issue, noted by Alam (2000) is not just one of registration, rather it is regulation and enforcement; registration alone does not prevent pirated products from being sold illegally.

The adoption of registration systems that fast-track biopesticides and take a pragmatic and flexible approach, including harmonizing registration requirements with other countries and acceptance of waivers and publicly available data, is a necessary step to improving the supply of biopesticides in many LICs (Grzywacz et al., 2009; East African Community, 2019). Biopesticides do not readily fit into a standard pesticide registration model so that registration systems in LIC require adaptation to fit the specific characteristics of biopesticides. There is evidence that the adoption of registration protocols favorable to biopesticides has greatly increased the supply of new products and stimulated the local biopesticide industry in some LIC (Cherry and Gwynn, 2007; Gwynn and Maniania, 2010; Mishra et al., 2020). Currently, Pakistan is developing a registration system for biocontrol products. "Aflatoxin control in Pakistan" is a project being run in Pakistan and the USA to develop a commercial biocontrol product—based on a nontoxin producing fungal strain of *Aspergillus flavus*—to reduce aflatoxins in maize. Through this project, not only has a nontoxin producing isolate been found and developed into a product called "AflaPak" for Pakistan. This project has also worked with the Pakistani authorities to develop a registration system for biocontrol products. Once registered, AflaPak will be the first-ever registered fungal native biocontrol product of its nature in Pakistan, opening opportunities for even more green technologies to be adopted in Pakistan (https://www.agrilinks.org/post/aflapak-registration-will-pave-way-enhance-livelihoods-maize-growers-pakistan).
Fundamentally, for some species, registration should be easy, based only on a detailed characterization of the isolate to be used; if these are known species of *Beauveria, Metarhizium* or *Trichoderma* this should be enough to allow sale and field use, unless product quality, purity and efficacy also fall within the purpose of registration in the particular country or region. For all other fungal species, a simplified acute oral and dermal test may be required. Various regulatory models are presented and discussed in Kabaluk et al. (2010), who note that different regions have different philosophies; South and Central America work with a belief that indigenous organisms are safer than the chemicals they replace, Cuba and parts of Asia prioritize the protection of consumers and farmers whilst encouraging local industries and Europe is employing Qualified Presumption of Risk for organisms from specific groups.

However, regardless of the registration system, existing small market sizes are serious constraints. In Africa and many parts of Asia and South America, national markets for all pest management products are small and this inhibits the registration and commercialization of all biopesticides. An example of this is the production of *Trichoderma stromaticum* for control of *Moniliopthera perniciosa*, which causes witches' boom disease in cacao. This species of *Trichoderma* is very host specific and is only associated with cacao and *M. perniciosa*. Successful local production was set up by a few growers, but the Brazilian authorities stopped local production, requiring it to be registered. Most of the cacao growers are small scale growers who cannot afford to buy commercial products unless crop price is high (Harman et al., 2010). There is a need to develop larger markets through harmonization of regulations and trading blocs (Cherry and Gwynn, 2007). It seems likely that biopesticides were able to become established commercially in Brazil, India and China partly because these countries represented large trading opportunities with many product niches, all accessible through a single regulatory system.

To try and address this issue in southern Africa, the Southern African Development Community (SADC), Southern African Pesticide Regulators Forum (SAPReF) was established in 2011. The role of the forum is to promote collaboration on pesticide and pest management, including regulation. Amongst their objectives are the promotion of less toxic alternatives to chemical and the promotion of regional collaboration and harmonization for pesticide regulation in the SADC region (Southern African Development Community, 2012). This concept received impetus when the International Center

for Genetic Engineering and Biotechnology (ICGEB) was recently granted funding by the Standards and Trade Development Facility (STDF) for a 3-year project to address this problem in southern Africa. The goal of the project is to promote the registration and use of biopesticides in the SADC region and hence reduce chemical pesticide residues in major export crops (International Center for Genetic Engineering and Biotechnology, 2021). This will be done through regional regulatory harmonization in the SADC region, residue mitigation and capacity development (Standards and Trade Development Facility, 2019). It is expected to increase the availability and use of biopesticides in the region, increase compliance with residue standards in key export markets and thus boost trade from the SADC region.

15.11.3 Responsibility

Although registration should be quick, transparent, low cost and easy, subsequently there needs to be greater emphasis on the quality and efficacy of the products. Regulation should be very stringent to ensure that products are of the specified quality and meet label specifications and all products in use should be tested regularly. This must be effective enough that users become confident to apply the products. Finally, the responsibility of manufacturers for their products should be comprehensive and stringent. Producers must be responsible for significant problems, including health issues and deviations from label claims; a simple and cheap registration process should not allow producers to avoid responsibilities for their products. Unfortunately, it is unrealistic to expect regulators in LIC to have the capacity to ensure that product quality and efficacy are maintained after registration. Regional or national biocontrol industry associations should be formed to establish acceptable industry norms for product quality and efficacy and to self-police, as has happened in South Africa with the formation of SABO (Hatting et al., 2019). Similar harmonization efforts have also moved forward in the East African region spearheaded by the East African Community.

Government/public support is necessary to allow biopesticide businesses to establish. This is clear at research levels, to optimize products, support biopesticide businesses and for creating a commercial environment where biopesticides are viewed positively for their many advantages (Marrone, 2007). In this context it is notable that in India, the Department of Biotechnology, some state governments, and the Indian Council Agricultural Research have worked to implement a range of pro-biopesticide policies including low-cost provisional registration and supported biopesticide research and IPM programmes that promote biopesticides to farmers; these efforts have facilitated EP industry start-up and expansion (Rabindra and Grzywacz, 2010; Kumar et al., 2019; Mishra et al., 2020).

15.11.4 Future

Production of EP in LIC as illustrated by the cases of fungal EP, and to a lesser extent BV, is now established in parts of Asia, Africa and South America. However, production is not yet on the scale that is required to meet the needs of agriculture over the next decade as the use of chemical pesticides becomes increasingly restricted. Pesticide review programs, increased stringency in the regulation of MRLs, arbitrary retailer residue restrictions, and growing public sentiment are contributing to this pressure on chemical pesticides (Lacey et al., 2015; Moore, 2021). Although most of this pressure is mounting within the EU, similar directives and requirements might be expected for agricultural produce being imported into the EU, including from LIC. Additionally, other countries in the world may well follow the EU's lead.

While many of the producers have started with a "low technology production" deemed "appropriate" for LIC countries, this approach may be a key constraint to expanding production to the scale needed. Although it is possible to produce high-quality material using simple systems, almost invariably sophistication of knowledge and equipment is required in maintaining a pure stock culture, ensuring sterility, achieving a safe harvest, and ensuring a good shelf-life. Despite the various models of production and local production from micro-producers that can be profitable (and hence sustainable), the potential need to replace chemical pesticides on major field crops if legislation further restricts the use of chemical pesticides will require mass production of EP on the industrial scale. For some types of products such as several fungal EP, good mass production systems with a comprehensive knowledge base capable of supporting large scale expansion already exist, although knowledge of these has not yet spread widely to producers in LIC. However, for other promising agents, such as BV, reliable large scale production systems remain to be further developed. For all EP it is not just the adoption of good production norms but the rigorous application of effective QC that is the vital component for success.

Any increase in the adoption of EP requires a shift in thinking away from seeing EP products only as straight substitutes for chemical pesticides. They should be seen as components in holistic ecologically sound IPM systems (Glare and McKinnon, 2020; Moore and Jukes, 2020; Shapiro-Ilan et al., 2020). This approach has already appeared in the export horticulture sector of some LIC where MRL requirements and consumer pressure have driven the adoption of IPM.

Despite these constraints, good biological pesticides can be more effective, economic, and more environmentally sustainable than chemical insecticides and their use in LIC is increasing. The changing face of global agriculture is opening new niches, as well as broad-acre markets for EP, and this offers new opportunities for farmers in LIC to produce EP and use them. To achieve success, however, governments need to enact and implement new pro-biopesticide policies that facilitate EP production, registration, and use.

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Section III

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Chapter 16

Potential and challenges for the use of insects as feed for aquaculture

Laura Gasco[1], Ilaria Biasato[1], Paula Enes[2,3] and Francesco Gai[4]

[1]*Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco, Italy,* [2]*CIIMAR—Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Matosinhos, Portugal,* [3]*Department of Biology, Faculty of Sciences of University of Porto, Porto, Portugal,* [4]*Institute of Sciences of Food Production, National Research Council, Grugliasco, Italy*

16.1 Introduction

The *State of World Fisheries and Aquaculture 2020* report (FAO, 2020) indicates in 2018 a global fish production of 179 million tons (=metric tons), of which aquaculture accounts for 46% of the total and for 62% of the sale value (US $250 billion). Compared to the other food-supply sectors, the last fifteen years have seen aquaculture as the leading sector for growth rate (+5.8% from 2000 to 2016) and, considering the stagnation of fisheries, aquaculture has become the only solution to face the expected increasing consumer demand for fish products. Data projections indicate that by 2030 aquaculture will provide from 50% to 60% of the global fish supply (Béné et al., 2015; IPIFF, 2019a), while today aquaculture already covers over 50% of fish production for food purposes.

For many years, fish nutritionists considered a fish meal and fish oil as "Gold standards" for diet formulation (Turchini et al., 2019). Fish meal represented the "Golden protein" due to its high protein content (up to 72%), its coverage of fish needs for essential amino acids supply, its high palatability, the absence of antinutritional factors, and, at that particular time, its relatively low and affordable price. Fish oil, extracted during the fish meal production process, has always supplied cultured fish with phospholipids and great amounts of omega-3 (n3) long-chain polyunsaturated fatty acids (PUFA). For a long, aquaculture has been pointed out as responsible for the over-exploitation of wild marine resources, thus attracting criticisms and concerns (Jones et al., 2015). These concerns, together with the restricted amount of wild captures for feed purposes and the fast increase in fish meal price, have progressively led to great efforts in improving aquafeed formulations and finding alternative protein and lipid sources (Vongvichith et al., 2020).

As far as the protein fraction is concerned, a large amount of plant proteins and terrestrial processed animal proteins are nowadays used in aquafeeds in addition to fisheries by-products meals (Gasco et al., 2020a), thus maintaining aquaculture growth and sustainability (Oliva-Teles et al., 2015; Daniel, 2018). Plant proteins, such as soybean meal, rapeseed meal, corn or gluten meals, are largely available at a relatively low cost and have a good protein content, in particular when protein concentrates are considered. On the other hand, their utilization in carnivorous fish species raises several issues: essential amino acid deficiencies, absence of important components such as taurine and hydroxyproline, presence of antinutritional factors, possible contamination with mycotoxins, low palatability, and gut inflammations problems (Gasco et al., 2018a; Hua et al., 2019; Oliva-Teles et al., 2015; Turchini et al., 2019; Wang et al., 2017; Yin et al., 2018). The strategies applied to mitigate these issues have not fully succeeded yet (Castillo and Gatlin, 2015; Lee et al., 2020). Another issue of recent attention is the unsustainability of plant proteins due to their increased pressure on the environment (land use, deforestation, water) and the competition with the food and biodiesel sectors (Fry et al., 2016; Kim et al., 2019; Aragão et al., 2020; Parisi et al., 2020).

Fishery and aquaculture by-products have great potential as feed ingredients, but they have not been fully exploited yet (Šimat et al., 2019; Tarhouni et al., 2019; Gasco et al., 2020a). The processed animal proteins (such as poultry by-products and feather meals, pork products blood meals and meat, and bones meals) have protein levels ranging from 55% to up to 90% (Gasco et al., 2018b) with a suitable essential amino acid content, are cost-effective, and represent a good by-product valorization (Moutinho et al., 2017; El-Husseiny et al., 2018). Nevertheless, the European Union (EU)

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only allows in aquafeeds the use of Category 3 processed animal proteins from nonruminants, thus remarkably restricting the potential use of these resources (Kim et al., 2019).

Considering the lipid fraction, in addition, to providing essential fatty acids, which are fundamental components of cell membranes and promote the absorption of lipophilic nutrients (Halver, 2002), lipids are also a good source of energy to meet fish metabolism requirements, thus sparing proteins from being used as an energy source. Even if some marine lipid sources [like krill oil, copepod oil, and salmon (by-product) oil] could be used as alternatives to fish oil, the most commonly used lipids in aquafeeds are plant oils (such as linseed oil, rapeseed oil, soybean oil), as reviewed by Tocher (2015). These oil sources are largely available and cost-effective (Turchini et al., 2011; Sánchez-Moya et al., 2020), even if their production rise environmental concerns (van Zanten et al., 2015).

Lipids have their specific fatty acid profile which directly affects the quality of the aquaculture final product. In particular, the lack of long-chain-PUFA in fish oil alternatives leads to a less valuable nutritional fish lipid profile for human consumption. Furthermore, high levels of plant oils replacing fish oil can compromise fish growth by suppressing immune function (Du et al., 2017), inducing oxidative stress and liver damage (Chen et al., 2020), and stimulating inflammatory responses (Mu et al., 2018; Tan et al., 2019).

In recent years, insects have gained increasing attention as emerging and promising raw materials to solve the shortage of proteins in feed (Gasco et al., 2019a, 2020a; Sogari et al., 2019; van Huis, 2020a; Hua, 2021). The number of studies published on the use of insects as food and feed has exponentially increased, thus highlighting the remarkable interest in this topic (van Huis, 2020b). Indeed, in 20 years, the number of papers on this topic increased by about 4250%, ranging from six papers in 2000 to 253 in 2021. Furthermore, for 2022, already twenty-two papers show up in the Web of Knowledge when choosing "Edible insects" (all categories mixed) as keywords (Fig. 16.1).

Studies on insect-derived products as feed ingredients mainly aimed to assess their nutritional value for fish and terrestrial livestock (Khan, 2018; Lock et al., 2018; Biasato et al., 2019; Gasco et al., 2019c; Koutsos et al., 2019; Nogales-Mérida et al., 2019). Other important aspects such as the environmental (Arru et al., 2019; Cadinu et al., 2020) and socio-economic (Abro et al., 2020) impacts, and consumer perception (Mancuso et al., 2016; Ferrer Llagostera et al., 2019; Gasco et al., 2019b) were also considered. In 2017, the EU allowed the inclusion of insect-derived protein from seven insect species in aquafeeds [Reg. (EU) 2017/893]. Insects will not be the miracle cure for the protein shortage issue, but insect-derived processed animal proteins may have a central role when carnivorous fish species are concerned, due to the high protein requirements of these species (Nogales-Mérida et al., 2019). The use of insect processed animal proteins also shows positive effects on fish health due to the antimicrobial/prebiotic effects of bioactive compounds such as chitin, lauric acid or antimicrobial peptides (Biasato et al., 2018; Gasco et al., 2018a; Henry et al., 2018a, b; Antonopoulou et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Gasco et al., 2020c). At the same time, when insects are reared on organic streams, they have real benefits for the environment in terms of land and water uses, and greenhouse gas emissions (Bosch et al., 2019; Smetana et al., 2019; Gasco et al., 2020b).

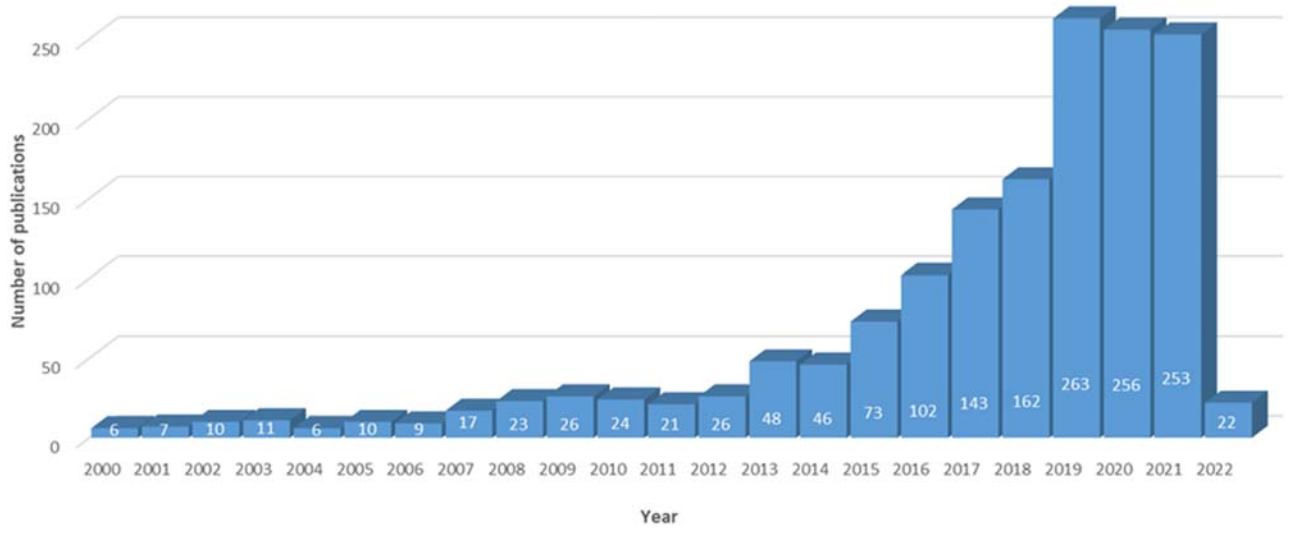

FIGURE 16.1 Number of "edible insects" published papers from 2000 to 2022.

Insects, particularly those having a larval stage, are rich in fat, representing the second-largest fraction of their composition (Danieli et al., 2019; Nogales-Mérida et al., 2019). During the insect-derived processed animal protein production, fat is partially extracted (Ravi et al., 2019, 2020; Hong et al., 2020) and can be used in animal nutrition as an energy source (Belghit et al., 2018; Cullere et al., 2019; Gasco et al., 2019c; Benzertiha et al., 2020; Sypniewski et al., 2020; Xu et al., 2020) or as biodiesel (Nguyen et al., 2019). Insects' lipid content and composition may change depending on the species and the life stage, as well as the biotic and abiotic factors (Liland et al., 2017; Meneguz et al., 2018; Smets et al., 2020).

Great attention has recently been paid to insect fat as bioactive compound, due to its richness in medium-chain fatty acids, such as lauric acid, that can have a positive effect on animal health in terms of gut microbiota modulation and immune response stimulation (Gasco et al., 2020c; Mouithys-Mickalad et al., 2020; Sypniewski et al., 2020).

This chapter reviews and reports a synthesis of the available literature about the potential of using insect-derived products in aquafeeds, with a focus on fish performance and health. It also highlights challenges to be solved to fully enable the real inclusion of these products in aquafeeds.

16.2 Insects in aquafeeds: performances and digestibility

The performance and diet digestibility of fish fed insect-derived products have recently been reviewed by Gasco et al. (2019a), Nogales-Mérida et al. (2019), and Hua (2021). Therefore, this subchapter only briefly reports the main results obtained in species of major economic interest for aquaculture.

16.2.1 Insect proteins: effects on fish meal and soybean meal sparing

Performance and diet digestibility studies using insect-derived products in aquafeeds resulted in divergent conclusions, mainly because of the variability of the nutritional value of insect meal, and/or the different nutritional requirements of fish species and life stages.

The most commonly investigated insect species are the black soldier fly (*Hermetia illucens* L., BSF) and the yellow mealworm (*Tenebrio molitor* L.) as these two species are the most promising candidates for mass production (Cadinu et al., 2020; Ravi et al., 2020; Rumbos et al., 2020). Growth performance, nutrient digestibility and product quality traits were the most important parameters investigated in studies performed with commonly cultured freshwater and marine fish species. The main results obtained are summarized in Table 16.1.

Multiple studies conducted on freshwater species showed the great potential of an insect as a partial or total substitute for dietary fish and soybean meals. In rainbow trout (*Onchorynchus mykiss* Walbaum), one of the most studied freshwater species, Sealey et al. (2011) included 16.4% and 32.8% of full-fat fish offal-enriched BSF prepupae meal to partially (25% and 50%) substitute fish meal obtained similar growth performances compared to the ones of the anchovy meal based-diet. Other studies, also in rainbow trout (initial body weight of 46, 67, or 179 g), showed that diets containing 26%, 28%, and 40% of defatted BSF meal in substitution of 13%, 46%, or 50% of fish meal respectively, did not impair survival, growth and feed conversion ratio (Stadtlander et al., 2017; Renna et al., 2017; Dumas et al., 2018). In contrast, significant and improved final body weight, feed conversion rate and specific growth rate were reported in Eurasian perch (*Perca fluviatilis* L) fed diets containing 20% and 40% of partially defatted BSF meal compared to fish fed 60% of BSF meal inclusion (Stejskal et al., 2020).

In rainbow trout fed a diet including 20% of BSF meal in substitution of the basal diet formulated for digestibility purposes, Dumas et al. (2018) found comparable results for apparent digestibility coefficients (ADCs) of crude protein (CP) and dry matter (DM), but lower for ether extract than the ones reported by Renna et al. (2017), Dumas et al. (2018) argued that the low lipid digestibility observed at high BSF inclusion levels could have been related to BSF meal chitin content. However, the same authors also suggested a possible positive effect of chitin on prolidase activity that can support the higher hydroxyproline and tryptophan ADCs found in fish fed BSF meal diets compared to the control group. On the contrary, Caimi et al. (2020b) showed lower ADC of CP (86.5% and 86.6%, respectively) when compared to a fishmeal based diet (88.5%) in a study carried out in Siberian sturgeon (*Acipenser baerii* Brandt) juveniles fed diets with 25% and 50% of a highly defatted BSF meal (CP: 62.5%).

Research also investigated ADCs of diets including *T. molitor* meal. For instance, Belforti et al. (2015) feeding rainbow trout with diets containing 25% and 50% of a full fat *T. molitor* meal reported lower ADC of CP in fish fed 50% of *T. molitor* meal, while DM, organic matter and ether extract ADCs were unaffected. The authors also reported a general negative impact of the highest *T. molitor* meal inclusion on growth parameters. Recently, in rainbow trout fed diets including partially defatted *T. molitor* meal at 5%, 10%, and 20%, Chemello et al. (2020) found a decrease in the ADC

TABLE 16.1 Insect proteins: effects on fish meal/soybean meal protein replacement.

Fish species/weight (g)/days of feeding	Insect species	Insect form	% Insect meal inclusion	Max %FM substitution	Results	References
Atlantic salmon (*Salmo salar*)/1400/114	HI	Larva meal	4.91%, 9.84%, and 14.75%	100% (FM)	→ ADC for protein, lipid and EAAs → feed intake, daily growth increase and FCR ↗ rancid odor	Belghit et al. (2019)
Atlantic salmon (*Salmo salar*)/49/56	HI	Larva meal	60%	83% (FM)	↘ ADC for protein, lipid and AAs (HI 60%)	Belghit et al. (2018)
Atlantic salmon (*Salmo salar*)/247/105	HI	Larva meal	5%, 15%, and 25%	100% (FM)	→ sensory parameters	Lock et al. (2016)
Blackspot seabream (*Pagellus bogaraveo*)/ 110–160–246/131	TM	Larva meal	21% and 40%	49% (FM)	→ water holding capacity and texture characteristics ↗ redness index in the skin (TM 40%)	Iaconisi et al. (2017)
Eurasian perch (*Perca fluviatilis*)/22/84	HI	Larva meal	20%, 40%, and 60%	62,5% (FM)	↘ final body weight and specific growth rate (HI 60%)	Stejskal et al. (2020)
European seabass (*Dicentrarchus labrax*)/ 50/62	HI	Pre-pupa meal	6.5%, 13%, and 19.5%	45% (FM)	→ growth performances	Magalhães et al. (2017)
European seabass (*Dicentrarchus labrax*)/ 5/70	TM	Larva meal	25%	71% (FM)	↗ ADC for protein	Gasco et al. (2016)
European seabass (*Dicentrarchus labrax*)/ 12/56	HI	Larva meal	7.25%, 10.1%, and 14.8%	45% (FM)	→ fish growth, feed utilization and survival	Abdel-Tawwab et al. (2020)
European seabass (*Dicentrarchus labrax*)/ 6/84	MD HI TM	Larva meal	19.5%	30% (FM)	↗ FCR (TM)	Mastoraki et al. (2020)
Gilthead seabream (*Sparus aurata*)/105/163	TM	Larva meal	25% and 50%	71% (FM)	↘ ADC for protein (TM50%) → water holding capacity and texture characteristics	Piccolo et al. (2017)
Meagre (*Argyrosomus regius*)/18/63	HI	Larva meal	10%, 20%,% and 30%	52% (FM)	↘ ADC of dry matter, energy, protein and some EAAs	Guerreiro et al. (2020a, b)
Rainbow trout (*Onchorynchus mykiss*)/ 179/78	HI	Larva meal	25% and 50%	50% (FM)	→ survival, growth and feed conversion ratio ↗ DM and EE contents in dorsal fillets (HI 40%) ↗ metallic flavor	Renna et al. (2017), Borgogno et al. (2017)

(*Continued*)

TABLE 16.1 (Continued)

Fish species/weight (g)/days of feeding	Insect species	Insect form	% Insect meal inclusion	Max %FM substitution	Results	References
Rainbow trout (*Onchorynchus mykiss*)/ 46/84	HI	Larva meal	26%	100% (FM)	→ survival, growth and feed conversion ratio ↘ ADC for lipid and dry matter ↗ ADC for hydroxyproline and thryptophan	Dumas et al. (2018)
Rainbow trout (*O. mykiss*)/78/154	TM	Larva meal	5%, 10%, and 20%	100% (FM)	↘ ADC for crude protein (TM20%) → growth parameters	Chemello et al. (2020)
Rainbow trout (*Onchorynchus mykiss*)/ 116/90	TM	Larva meal	25% and 50%	67% (FM)	↘ ADC for crude protein (TM50%) → ADC for dry matter, organic matter and ether extract ↘ growth parameters → moisture, protein and ash fillet content ↘ overall significant reduction in the fillet EAA (TM50%)	Belforti et al. (2015), Iaconisi et al. (2018; 2019)
Siberian sturgeon (*Acipenser baerii*)/24/ 118	HI	Larva meal	25% and 50%	50% (FM)	↘ ADC for crude protein	Caimi et al. (2020a)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the insect meal diet relative to the control. In the case of insect meal being tested at more than one level, and when not indicated, it means that all levels had the same effect. *ADC*: apparent digestibility coefficient; *EAA*, essential amino acid; *EE*, eher extract; *FCR*, feed conversion rate; *FM*, fish meal; *HI*, Hermetia illucens; *MD*, *Musca domestica*; *TM*, Tenebrio molitor.

of CP, while the growth parameters were not affected, thus contradicting data reported by Belforti et al. (2015). The lower *T. molitor* meal dietary inclusion used in the Chemello et al. (2020) trial or, most likely, the inclusion of essential amino acids leading to diets that better fit the fish requirements can explain these contrasting results between studies.

Another important aspect investigated is related to the quality of the derived food product (such as fish fillet) in terms of proximate composition, color, amino acid and fatty acid profiles, and sensorial perception.

In rainbow trout raw and cooked fillets, Iaconisi et al. (2018) found that *T. molitor* larva meal based-diets did not lead to changes in moisture, protein or ash contents, even if high dietary inclusion levels (up to 50%) were used. On the other hand, when *T. molitor* meal was included at 50%, the amino acid profile of rainbow trout fillet was affected. The major changes emerged in terms of total and free amino acid composition, with an overall significant reduction in the essential amino acids alanine, isoleucine, leucine, and lysine (Iaconisi et al., 2019). On the contrary, Renna et al. (2017) observed an increased DM and ether extract contents in rainbow trout dorsal fillets of fish fed the highest level (40%) of BSF larva meal dietary inclusion. Organoleptic properties of fish fillets are also important parameters that were evaluated in several nutritional trials carried out on rainbow trout. Generally speaking, no differences are highlighted by common consumers. Indeed, no significant differences were found in terms of taste and odor in BSF meal-fed fish by untrained panelist as reported by Stadtlander et al. (2017), while significant changes, even if small, in the odor, flavor, color and texture characteristics were identified by trained panelist as described by Borgogno et al. (2017).

Looking at works carried out in marine fish species, a consistent number of studies were performed in gilthead seabream (*Sparus aurata* L.), European seabass (*Dicentrarchus labrax* L.), and Atlantic salmon (*Salmo salar* L.), but also few recent studies on emerging fish species such as meagre (*Argyrosomus regius* Asso) are now available. Regarding digestibility trials, controversial results were found based on the type of insect meal and fish size used.

Compared to fish meal-based diets, lower ADC of CP (79.19% vs 89.97) was recorded for large gilthead seabream (above 230 g) fed a diet containing 50% of full-fat *T. molitor* (Piccolo et al., 2017). In contrast, dietary inclusion of 25% of the same insect meal in European seabass juveniles, led to a significantly higher ADC of CP (0.92 vs 0.90) than that of the fish fed the fish meal-based diet (Gasco et al., 2016). In Atlantic salmon, 60% of BSF meal dietary inclusion, led to a reduction of the ADC of CP, lipid and all the investigated amino acids (Belghit et al., 2018), while in sea-water phase Atlantic salmon the ADCs of CP, lipid, amino acids or fatty acids were not affected with the inclusion of BSF meal at 4.9%, 9.8% and 14.8% (Belghit et al., 2019). These authors found comparable amino acids digestibility values with those observed by Magalhães et al. (2017) in European seabass fed BSF meals. However, Guerreiro et al. (2020a) reported that ADCs of DM, energy, protein and some essential amino acids such as lysine, isoleucine, leucine, phenylalanine, and alanine, decreased with increased BSF meal inclusion levels (10%, 20% and 30%) in meagre diets.

As far as fish growth parameters are concerned, most of the feeding trials that have been performed in several species concluded that dietary inclusion of BSF meal up to 20% is feasible without negative effects on growth performance or feed conversion ratio. Thus, European sea bass juveniles fed defatted BSF meal diets, with a maximum inclusion level of 14.8%, showed similar growth, feed utilization and survival to the ones fed with a fish meal-based diet (Abdel-Tawwab et al., 2020). Magalhães et al. (2017) and Mastoraki et al. (2020) also showed that a 19.5% of BSF meal inclusion can be used in diets of juvenile European seabass without impairing growth performances. Belghit et al. (2019) reported similar levels of inclusion, up to 15%, of BSF meal in diets for sea-water phase Atlantic salmon, whereas in Atlantic salmon with a final body weight of around 3.7 kg, the partial or total replacement of fish meal with BSF meal was feasible without compromising ng feed intake, daily growth increase or feed conversion ratio.

On the other hand, meagre juveniles growth, feed efficiency and protein efficiency ratio linearly decreased with the increase (10%, 20% and 30%) of dietary BSF meal levels (Guerreiro et al., 2020b).

In European seabass juveniles, dietary inclusion of full-fat *T. molitor* meal at 50%, without an appropriate balance of essential amino acids, led to poor performance results (Gasco et al., 2016). Also in European seabass juveniles, a full-fat *T. molitor* meal dietary inclusion level of 19.5% resulted in a slight increase in feed conversion ratio compared to a fish meal control group and two other fish groups fed *H. illucens* or *Musca domestica* (Mastoraki et al., 2020).

Product quality parameters were also investigated in marine fish trials using dietary *T. molitor* larva meal. *T. molitor* larva meal dietary ingestion did not influence the water holding capacity or the texture characteristic of gilthead seabream and blackspot seabream fillets (*Pagellus bogaraveo*) (Iaconisi et al., 2017; Piccolo et al., 2017). In the same works also skin color parameters were investigated. Thus, blackspot seabream fed the maximum *T. molitor* larva meal dietary inclusion level (40%) showed a higher redness index (a*) in the ventral region, while gilthead seabream did not show any modifications independently of the insect meal inclusion level tested.

In fish products sensory properties, such as aroma and flavor, which are closely related to the dietary lipid-volatile components, may also be affected by dietary insect meal inclusion (Borgogno et al., 2017). Even if not much data are available on marine fish, a study on Atlantic salmon (final body weight of about 550 g) (Lock et al., 2016) fed 5% or 10% of BSF larva meal inclusion revealed that trained panelists were not able to perceive any differences in the sensory parameters of cooked fillets. On the other hand, small but significant changes in the fillet sensory qualities (odor, flavor, color, and texture characteristics) were found in the baked fillets of commercial size Atlantic salmon fed the highest dietary inclusion level (14.8%) of BSF larva meal (Belghit et al., 2019). Therefore, the authors concluded that using insect ingredients in Atlantic salmon feeds leads to only marginal changes in fish fillet sensory quality.

16.2.2 Insect fat and oils: effects on fish and soybean oil sparing

Although fish oil has a valuable fatty acid content, due to global increasing costs and limited availability (Turchini et al., 2019), the aquafeeds industry largely uses alternative dietary lipid sources, mainly of plant origin (Tocher, 2015; Turchini et al., 2019). However, as mentioned before for plant proteins, plant oil obtainment has also negative environmental impacts (van Zanten et al., 2015; Benzertiha et al., 2020).

Compared to research performed on insect meal protein, a lower number of studies investigated the use of insect fat/oil in fish nutrition (Benzertiha et al., 2020). Table 16.2 summarizes the main results obtained in trials where insect fat/oil spared fish or soybean oils.

Generally speaking, the inclusion of BSF fat in the substitution of fish or soybean oils did not impaired growth, feed utilization or somatic indices in different fish species such as Jian carp (*Cyprinus carpio* L. var Jian) and mirror carp (*C. carpio* var specularis) (Li et al., 2016, Xu et al., 2020), Atlantic salmon (Belghit et al., 2018), and rainbow trout (Dumas et al., 2018).

TABLE 16.2 Insect fat and oil: effects on fish oil/soybean oil replacement.

Fish species/weight (g)/days of feeding	Insect species	Insect form	Feed composition	% Insect oil inclusion	% FO-VO substitution	Results	References
Atlantic salmon (*Salmo salar*)/≈49/56	*Hermetia illucens*	Larvae Fat	FO-RO based diet	12	100 (RO)	→Whole-body protein, lipid, amino acids and minerals contents	Belghit et al. (2018)
Jian carp (*Cyprinus carpio var Jian*)/≈33/59	*Bombyx mori*	Chrysalis Oil	SBO based diet	25, 50, 75 and 100	Up to 100 (SBO)	↘ hepatopancreas lipid content at 50 SBO replacement level ↗ muscle crude protein content at 50 SBO replacement level → serum biochemical indices and hepatopancreatic malondialdehyde content	Chen et al. (2017)
Jian carp (*Cyprinus carpio var Jian*)/≈10/59	*Hermetia illucens*	Larvae Fat	SBO based diet	6.25,12.5, 18.75 and 25	Up to 100 (SBO)	↘ intraperitoneal fat and adypocite size at higher SBO replacement levels ↗ PPARα relative gene expression → PPARγ and FAS relative gene expression	Li et al. (2016)
Mirror carp (*Cyprinus carpio* var specularis) ≈ 14/59	*Hermetia illucens* *Tenebrio molitor* *Bombyx mori*	Larvae Fat	FM, SBM, RSM based diet	2.5 (BSFO) 2.5 (YMO) 2.5 (SWPO)	No FO or SBO replacement	↘ intraperitoneal fat and adypocite size in BSFO and MIXO diets ↗ PPARα relative gene expression in BSFO and MIXO diets ↗ hepatic expression of pro-inflammatory cytokine (IL-1β) and tumor necrosis-α (TNF-α) in SWPO	Xu et al. (2020)
Rainbow trout (*Onchorynchus mykiss*)/≈46/90	*Hermetia illucens*	Larvae Fat	FO-based diet	2.5, 5.0, and 10	25, 50, and 100 (FO)	→ relative body protein content → histology of the posterior intestine ↘ blood glucose at the 50% and 100% FO replacement levels ↗ digestibility of hydroxyproline	Dumas et al. (2018)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the insect meal diet relative to the control. In the case of insect meal being tested at more than one level, and when not indicated, it means that all levels had the same effect. *BSFO*, black soldier fly oil; *FAS*, fatty acid synthase; *FM*, fish meal; *FO*, fish oil; *MIXO*, mixture of SFO, SWPO and YMO; *PPARα*, Peroxisome proliferator-activated receptor alpha; *RSM*, rapeseed meal; *RO*, rapeseed oil; *SBM*, soybean meal; *SBO*, soybean oil; *SWPO*, silkworm pupae oil; *YMO*, Yellow mealworm oil.

Otherwise, only a few studies are available on the use of silkworm (*Bombix mori* L.) oil as reported in Jian carp (Chen et al., 2017) and mirror carp (Xu et al., 2020). Growth performance and feed utilization indices as well as somatic indices were not affected by the inclusion of silkworm oil, even if the levels of feed intake suggested that at moderate inclusion levels, silkworm oil was slightly better accepted compared to control diets. A hypothesis could be the presence of an attractant effect of insect lipids for some fish species as reported by Kasumyan, 2018. Similar results on voluntary feed intake, final body weight, feed conversion ratio, protein efficiency ratio and animal condition were observed in Atlantic salmon fed with BSF fat from larvae grown on organic waste streams or on seaweeds, as a total replacement of rapeseed oil alone or with BSF meal supplementation (Belghit et al., 2018). However, the diet containing BSF fat from larvae grown on organic waste streams decreased Atlantic salmon's daily growth index and specific growth ratio in comparison to a rapeseed and fish oil control diet. In another trial with rainbow trout, a BSF fat mechanically extracted from larvae grown on undescribed feedstock was included in diets at 2.5%, 5%, and 10% to replace fish oil (Dumas et al., 2018). Again, growth performance, feed utilization parameters, and hepatosomatic index was not impaired.

As previously observed in studies carried out on fish oil replacement with plant oils, insect oil impacted the fatty acid profile of fish tissues, which mirrors diet fatty acid composition. The amounts of fatty acids as well as their profile in insects are dependent on different variables such as insects' stage of development, rearing conditions, and species (Barragan-Fonseca et al., 2017; Smets et al., 2020). Therefore, it is not surprising that the fatty acid profile obtained from BSF fat is quite different from that obtained by oil extracted from *T. molitor* or *B. mori*.

The BSF fatty acid profile is rich in saturated fatty acids and particularly in medium-chain fatty acids with lauric acid (C12:0) representing 21.4%−49.3% of the total fatty acids. BSF fat also contains linoleic acid (C18:2n-6; concentration 3.6%−4.5%) and α-linolenic acid (18:3n-3; 0.08%−0.74%) (Tran et al., 2015). The fatty acid composition of *B. mori* and *T. molitor* oils is dominated by monounsaturated fatty acids (MUFA) and PUFA, with oleic acid (C18:1n-9), linoleic and α-linolenic acids as the major contributing fatty acids (Wang et al., 2020; Wu et al., 2020).

Nutritional trials carried out with juvenile Jian carp showed that in terms of fatty acid composition, a 100% dietary inclusion of silkworm chrysalis oil, significantly increased hepatopancreas, intraperitoneal fat, and muscle omega 3 PUFAs and the n3/n6 ratios (Chen et al., 2017). On the contrary, Jian carp fed 12.5% and 18.75% of BSF larva fat showed an increase in the saturated fatty acid fraction of intraperitoneal fat tissue, muscle and hepatopancreas due to dietary rise in lauric and myristic acids contents (Li et al., 2016). To better understand the effects of insect oil and fats on fish lipid metabolism, some authors performed gene expression analysis of peroxisome proliferator-activated receptors (PPARs) in adipocytes isolated from intraperitoneal fat tissue. Li et al. (2016) found a decreased lipid deposition in Jian carp intraperitoneal fat tissue, coupled with a higher gene expression of PPARα, when fish were fed increasing levels of BSF fat in substitution of soybean oil. Xu et al. (2020) found similar results in mirror carp fed with BSF fat compared to fish fed other insect oils such as *B. mori* and *T. molitor* oils rich in MUFA and PUFAs. In fish fed BSF fat as the only fat source or in combination with other insect lipid sources, intraperitoneal fat index and adipocyte size decreased, while relative gene expression of PPAR-α significantly increased contrary to the fatty acid synthase gene expression that significantly decreased (Xu et al., 2020).

Overall, data available on the use of insect oils/fat as alternative lipid sources to fish and soybean oils are encouraging, even if from an economical point of view insect lipids are still generally more expensive than traditional lipid sources used in aquafeeds production.

16.3 Insects and fish health

16.3.1 Gut morphology

It is well known that the gut is the main site where nutrient digestion and absorption occur and its health status greatly influences the utilization of the dietary nutrients. Therefore, morphological changes in the gut are commonly investigated in fish when novel feed ingredients are tested. As a first aspect to consider, the morphometric features of selected gut elements (such as the mucosal villi or the muscular layer) are considered the main indicators of gut health and functional status (Elia et al., 2018). Indeed, a healthy gut is generally characterized by elongated villi and thick mucosa and muscular layers (Józefiak et al., 2019). Furthermore, the classic histological analysis may provide useful information in terms of immune cell infiltration within the mucosa and the submucosa (Elia et al., 2018), as well as shortening and fusion of Nakcosal folds, reduced enterocyte vacuolization, and nucleus position disparity (all typical inflammatory changes) (Li et al., 2020). Finally, the gut mucins, which are highly glycosylated glycoproteins synthesized by goblet cells and involved in the nutrient absorption, digestive efficiency, and defense against pathogens, also object of great

attention. Indeed, the utilization of specific histochemical stainings such as the Periodic Acid-Schiff and the Alcian Blue pH 2.5 allows their characterization and classification into neutral and acidic, respectively (Elia et al., 2018).

Despite the importance of the mentioned gut health parameters, few studies assessing the impact of insect feed ingredients on these parameters are currently available. Insect meal effects on fish gut morphology are reported in Table 16.3. In relation to the gut morphometric features, unaffected villus height was observed in rainbow trout (Renna et al., 2017; Dumas et al., 2018), and juvenile Siberian sturgeon (*Acipenser baerii*) (Józefiak et al., 2019; Caimi et al., 2020a) fed different inclusion levels of BSF meal (Renna et al., 2017; Józefiak et al., 2019; Caimi et al., 2020a), BSF oil (Dumas et al., 2018), or *T. molitor* meal (Józefiak et al., 2019) when compared to fish fed a fish meal-based diet. On the contrary, Chaklader et al. (2019) reported increased villi and enterocyte width, as well as microvilli height, in juvenile barramundi (*Lates calcarifer* Bloch) fed poultry by-product-based diets supplemented with 12% of BSF meal, thus indicating the enlargement of digestion and absorption surface area. A different scenario was highlighted by Józefiak et al. (2019), who observed higher muscular layer thickness in juvenile Siberian sturgeon fed 15% inclusion levels of *T. molitor* and BSF meal than those fed a control diet containing 26% of fish meal, as well as lower mucosa thickness in fish fed BSF meal-based diets when compared to the other dietary treatments. As a final aspect to underline, a significant reduction in the intestinal fold length was identified in juvenile clownfish (*Amphiprion ocellaris* Cuvier) fed 40% and 60% inclusion levels of BSF meal (Vargas-Abúndez et al., 2019), as well as in rainbow trout fed diets containing 10.5% and 21% of BSF meal (Cardinaletti et al., 2019). Similarly, the inclusion of 26.4% of BSF meal in diets for rainbow trout led to a shortening of the villi in the anterior gut (Dumas et al., 2018). Despite the observed negative alterations in the gut morphology (in terms of thinner mucosa and shorter folds), the growth performance of the fish resulted unaffected in the majority of the cases (Cardinaletti et al., 2019; Józefiak et al., 2019; Vargas-Abúndez et al., 2019), thus suggesting no remarkable effects of insect meal utilization on the intestinal absorption surface.

Recent studies assessing the gut histological changes in fish after the administration of insect-based diets reported similar immune cell infiltration within the gut mucosa and submucosa in both the BSF- and fish meal-fed rainbow trout (Elia et al., 2018) and juvenile Siberian sturgeon (Caimi et al., 2020a). On the contrary, Li et al. (2020) observed less severe enterocyte hypervacuolization (a potential, histological marker of lipid malabsorption) and a higher degree of submucosa cellularity (potentially indicative of gut inflammation) in Atlantic salmon fed a diet containing 14.8% of BSF meal when compared to a fish meal LT94 diet. However, the increased submucosa cellularity was not accompanied by a differential expression of the proinflammatory marker genes (Li et al., 2020). It is also important to underline that in all the above-mentioned studies the gut histological changes were not remarkable and were identified in the control fish as well, thus confirming that the use of insect meal does not induce the development of gut diseases.

Mucin staining intensity signaling unaffected gut mucin composition was reported in rainbow trout (Elia et al., 2018) and juvenile Siberian sturgeon (Caimi et al., 2020a) fed BSF-based diets in comparison with the fish meal (Chile, super-prime) based diet. Elia et al. (2018) also observed a predominance of neutral mucins over the acidic subtype in both the BSF- and the control-fed fish. Despite the scarcity of information about the gut mucin composition in rainbow trout, neutral mucins are known to promote nutrient absorption and transportation (especially carbohydrates and fatty acids) (Caimi et al., 2020a). A greater number of acidic mucins was also identified in the juvenile barramundi fed BSF-supplemented diets, thus protecting fish by binding and preventing the adherence of pathogenic bacteria to the gut epithelium (Chaklader et al., 2019).

16.3.2 Immune response

Nutrition is an important vehicle for immunomodulation in fish (Kiron, 2012). The concept of maintaining fish health through nutrition is well-accepted in fish farming and thus, special attention is given to the selection of the appropriate protein sources to be included in aquafeeds. Besides being considered one of the most promising sources of alternative proteins, insects are also sources of compounds able to exert positive effects on animals' immune systems, hence improving health status and decreasing susceptibility to diseases (Gasco et al., 2018a). However, few studies (Ogunji et al., 2008; Ming et al., 2013; Ido et al., 2015, 2019; Taufek et al., 2016; Su et al., 2017; Henry et al., 2018a, b; Sankian et al., 2018; Song et al., 2018; Xiao et al., 2018; Chaklader et al., 2019; Li et al., 2019; Stenberg et al., 2019; Fawole et al., 2020) have addressed the effects of fish meal replacement by insect meals in fish immune function (Table 16.4) and resistance against common pathogens (Table 16.5).

Hematological parameters such as white blood cells and red blood cells total counts, differential leukocyte counts (lymphocytes, neutrophils, basophils, eosinophils, and monocytes), hemoglobin (Hb), hematocrit (Ht), and erythrocyte indices including mean corpuscular volume (MCV), mean concentration hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are consider important tools to assess fish health status. The few studies available

TABLE 16.3 Insect meal effects on fish gut morphology.

Fish species	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Days of feeding	Results	References
Atlantic salmon (*Salmo salar*)	Partially defatted *Hermetia illucens*	Larva meal	FM, soy protein concentrate, pea protein concentrate, corn gluten and wheat gluten-based diet	14.75	100 (FM) and 68 (pea protein concentrate)	112	↘enterocyte hypervacuolization ↗submucosa cellularity	Li et al. (2020)
Barramundi (*Lates calcarifer*)	Full-fat *Hermetia illucens*	Larva meal	FM- and PBM-based diet	12	No substitution (supplementation)	42	↗villi and enterocyte width ↗microvilli height ↗acidic mucins	Chaklader et al. (2019)
Clownfish (*Amphiprion ocellaris*)	Partially defatted *Hermetia illucens*	Larva meal	FM-based diet	20, 40 and 60	25, 50 and 75	106	↘fold length (40 and 60)	Vargas-Abúndez et al. (2019)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Larva meal	FM-based diet	20 and 40	25 and 50	78	→Vh	Renna et al. (2017)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Larva meal and oil	FM- and FO-based diet	6.6, 13.2 and 26.4 (meal); 2.5, 5 and 10% (oil)	25, 50 and 100 (meal and oil)	84	↘Vh (26.4%)	Dumas et al. (2018)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Larva meal	FM-based diet	20 and 40	25 and 50	78	→mucins	Elia et al. (2018)
Rainbow trout (*Onchorynchus mykiss*)	Full-fat *Hermetia illucens*	Prepupa meal	FM, pea protein concentrate, and wheat gluten-based diet	10.5 and 21	25 and 50	98	↘fold length ↗neutral mucins (descriptive analysis)	Cardinaletti et al. (2019)
Siberian sturgeon (*Acipenser baerii*)	Full-fat *Hermetia illucens* and *Tenebrio molitor*	Larva meal	FM-based diet	15	29 (HI) and 40 (TM)	60	↘mucosal thickness (HI) ↗muscular thickness (TM) →Vh	Józefiak et al. (2019)
Siberian sturgeon (*Acipenser baerii*)	Highly defatted *Hermetia illucens*	Larva meal	FM- and PP-based diet	18.5 and 37.5	25 and 50	118	→Vh →mucins	(Caimi et al., 2020b)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the insect meal diet relative to the control. In the case of insect meal being tested at more than one level, and when not indicated, it means that all levels had the same effect. *FM*, fish meal; *FO*, fish oil; *HI*, Hermetia illucens; *TM*, Tenebrio molitor; *PBM*, poultry by-product meal; *PP*, plant protein; *Vh*, villus height.

TABLE 16.4 Insect meal effects on fish immune response.

Fish species/ weight (g)/ days of feeding	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Results	References
African catfish (*Clarias gariepinus*)/ ≈13/49	Full fat *Gryllus bimaculatus*	Adult meal	FM-based diet	26 and 35	75 and 100	→RBC, MCV; Hb, MCH and MCHC (26%) ↗Ht; Hb, MCH and MCHC (35%) ↘WBC	Taufek et al. (2016)
African catfish (*Clarias gariepinus*)/≈4/ 60	Partially deffated *Hermetia illucens*	Larvae meal	FM-based diet	6, 11.5, and 17	25, 50, and 75	→Ht, Hb, WBC, RBC, MCV, MCH, MCHC, neutrophil, lymphocyte and monocyte	Fawole et al. (2020)
Atlantic salmon/(*Salmo salar*)/49/56	*Hermetia illucens*	Larvae meal	FM + PF	60	85	→proximal and distal intestine gene expression of IL-4, TGFβ-1, IL-10, IFNγ, IL-8 and MYD88 ↗proximal and distal intestine gene expression of CD3γδ and FOXP3	Li et al. (2019)
European sea bass (*Dicentrarchus labrax*)/≈65/42	Full fat *Tenebrio molitor*	Larvae meal	FM based diet (control) TM diet; TM + proteases; TM + carbohydrases	25	36	→serum lysozyme; serum anti-Gram negative bacteriolytic activity in the TM and TM + carbohydrases; serum trypsin-inhibition in the TM + proteases and TM + carbohydrases ↗serum trypsin-inhibition at the TM ↘serum ceruloplasmin, myeloperoxidase and nitric oxide; serum anti-Gram negative bacteriolytic activity in the TM + proteases	Henry et al. (2018b)
Mandarin fish (*Siniperca scherzeri*)/≈21/ 56	Full fat *Tenebrio molitor*	Larvae meal	FM-based diet	10, 20, and 30	10, 20, and 30	→serum myeloperoxidase and total immunoglobulin; serum lysozyme (10% and 20%) ↗serum lysozyme (30%)	Sankian et al. (2018)
Nile tilapia *(Oreochromis niloticus)*/2/56	House fly (*Musca domestica*)	Larvae meal	FM-based diet	15, 25, 35, 45, 55, 68, and 100	21, 35, 49, 63, 76, and 100	→Ht; Hb (15%) ↗Hb (25%, 35%, 45%, 55%, 68% and 100%)	Ogunji et al. (2008)
Rainbow trout (*Oncorhynchus mykiss*)/≈116/ 90	Full fat *Tenebrio molitor*	Larvae meal	FM-based diet	25 and 50	35 and 67	→serum lysozyme, ceruloplasmin, nitric oxide, trypsin inhibition and antibacterial activity *Escherichia coli*; serum myeloperoxidase (25%) ↗serum myeloperoxidase (50%) ↘time for the killing of 50% *E. coli*	Henry et al. (2018a)

(*Continued*)

TABLE 16.4 (Continued)

Fish species/ weight (g)/ days of feeding	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Results	References
Yellow catfish (*Pelteobagrus fulvidraco*)/ ≈49/65	Full fat *Hermetia illucens*	Larvae meal	FM-based diet	5.5, 11, 16.5, 22, 34, 46, and 58.5	13, 25, 37, 48, 68, 85, and 100	↘phagocytic % (46% and 58.5%)	Xiao et al. (2018)
Yellow catfish (*Pelteobagrus fulvidraco*)/ ≈10/35	*Tenebrio molitor*	-	FM-based diet	9, 18, and 27	25, 50, and 75	→plasma lysozyme and complement 3; plasma IgM (9%) ↗plasma IgM (18% and 27%) →liver expression of MHC-II, CypA and HE; IgM (27%); IL-1β (18% and 27%) ↗liver expression of IL-1β (9%); IgM (9% and 18%) →spleen expression IL-1β and CypA; MHC-II (27%) ↗spleen expression IgM; MHC-II (9% and 18%) →kidney expression MHC-II, IL-1β, CypA, IgM and HE	Su et al. (2017)

Symbols represent no effect (→), an increase (↗) or a decrease (↘) in the response parameter of the insect meal diet relative to the control.
CD3γδ: XXXX; *CypA*, cyclophinlin A; FM, fish meal; FOXP3, forkhead box P3; Hb, hemoglobin; *HE*, hepcidin; *Ht*, hematocrit; *IFNγ*, interferon gamma; *IgM*, immunoglobulin M; *IL-1β, IL-4, IL-8, IL10*, interleukin-1β, 4, 8, 10; *MCH*, mean concentration hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume; MHC-II, major histocompatibility complex II; *MYD88*, myeloid differentiation primary response 88; *PF*, plant feedstuffs; *RBC*, red blood cell; *TGFβ-1*, transforming growth factor β-1; *TM*, *Tenebrio molitor*; *WBC*, white blood cell.

TABLE 16.5 Insect meal effects on fish disease resistance/challenge studies.

Fish species/weight (g)/days of feeding	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Challenge type/ dosage/duration	Results	References
Atlantic salmon (*Salmo salar*)/49/56	*Hermetia illucens*	Larvae meal	FM + PF	60	85	Intraperitoneal injection (0.1 mL) with inactivated whole viral vaccine based on highly immunogenic strains of IPNV	→plasma antibody level against IPNV	Li et al. (2019)
Atlantic salmon (*Salmo salar*)/ ≈1400/56	Partially defatted *Hermetia illucens*	Larvae meal	FM and PF based diet (20:80)	10 and 15	66 and 100	Head kidney leukocytes incubated with 100 μg/mL LPS from *Pseudomonas aeruginosa* and 50 μg/mL Poly I:C/24 h	→head kidney expression of IL-1β, IL-8, IL-10, TNF-α and TLR3 in both LPS and Poly I:C treatments; TLR22 in in Poly I:C treatment ↘head kidney expression of TLR22 in LPS treatment	Stenberg et al. (2019)
Barramundi (*Lates calcarifer*)/3.5/42	Full fat *Hermetia illucens*	Larvae meal	FM-based diet (control); 45% PBM + 10% HI and 90% PBM + 10% HI	10	-	Intraperitoneal injection with *Vibrio harveyi*/5.4×10^7 CFU/mL/24 h/14 days for survival	↗survival in the 45% PBM + 10% HI diet ↘survival in the 90% PBM + 10% HI diet →serum lysozyme and bactericidal activity in the 90% PBM + 10% HI diet ↗serum lysozyme and bactericidal activity in the 45% PBM + 10% HI diet →head kidney expression of complement 3 and 4 in the 90% PBM + 10% HI diet →head kidney expression of MHC-II ↗head kidney expression of complement 3 and 4 in the 45% PBM + 10% HI diet	Chaklader et al. (2019)

(*Continued*)

TABLE 16.5 (Continued)

Fish species/weight (g)/days of feeding	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Challenge type/ dosage/duration	Results	References
Pearl gentian grouper (*Epinephelus lanceolatus* ♂ x *Epinephelus fuscoguttatus* ♀)/≈7/50	Deffated *Tenebrio molitor*	-	FM-based diet	2.5, 5, 7.5, 10, and 12.5	6, 12, 18, 25, and 31	Intraperitoneal injection with *Vibrio harveyi*/2.79×10^9 CFU mL^{-1}/7 days	→survival (2.5%, 5% and 7.5%) ↘survival (10% and 12.5%) ↗RPS (7.5%) ↘RPS (10% and 12.5%)	Song et al. (2018)
Red sea bream (*Pargus major*)/≈30/56	Defatted *Tenebrio molitor*	Larvae meal	FM-based diet	5 and 10	10 and 20	Intraperitoneal injection with *Edwardsiella tarda*/5×10^6 CFU/mL/14 days	↗Survival, mainly at the 10%	Ido et al. (2019)
Red sea bream (*Pagrus major*)/≈48/10 for phagocytic activity of peritoneal leukocytes assay/48/60 for challenge	Full fat *Musca domesica*	Pupae meal	FM-based diet	0.75 and 7.5 for the phagocytic activity of peritoneal leukocytes assay; 5 for the challenge	-	Intraperitoneal injection with *Edwardsiella tarda*/5×10^6 CFU/mL/15	↗phagocytic activity of peritoneal leukocytes ↗survival	Ido et al. (2015)
Yellow catfish (*Pelteobagrus fulvidraco*)/≈10/35	*Tenebrio molitor*	-	FM-based diet	9, 18, and 27	25, 50, and 75	Intraperitoneal injection with *Edwardsiella ictaluri*/1 x 10^6 CFU/mL/24 h/14 days for survival	→plasma complement 3 and IgM ↗plasma lysozyme →liver expression of CypA and IgM; MHC II and IL-1β (18% and 27%); HE (9% and 27%) ↗liver expression of MHC-II and IL-1β (9%); HE (18%) →spleen expression of MHC-II, CypA and IgM; IL-1β (9% and 27%) ↗spleen expression of IL-1β (18%) →kidney expression of IL-1β, IgM and HE; CypA (9% and 27%) ↗kidney expression of MHC-II and CypA (18%) ↗survival in the 75% FM replacement level from day 7 to day 14	Su et al. (2017)

Symbols represent no effect (→), an increase (↗) or a decrease (↘) in the response parameter of the insect meal diet relative to the control.
CFU, colony forming units; *CypA*, cyclophinlin A; *FM*, fish meal; *HE*, hepcidin; *HI*, *Hermetia illucens*; *IgM*, immunoglobulin M; *IL-1β*, *IL-8*, *IL-10*, interleukins 1β, 8, and 10; *IPNV*, infectious pancreatic necrosis virus; *LPS*, lipopolysaccharide; *MHC-II*, major histocompatibility complex II; *PBM*, poultry by-product meal; *PF*: plant feedstuffs; *Poly I*: C, polyinosinic acid: polycytidylic acid; *RPS*, relative per cent survival; *TLR3, TLR22*, toll-like receptor 3 and 22; *TNF-α*, tumor necrosis factor α.

showed an absence or a positive effect of dietary insect meals on fish hematological parameters (Ogunji et al., 2008; Taufek et al., 2016; Fawole et al., 2020). Thus, inclusion of 6%, 11.5% and 17% of BSF meal in diets for African catfish (*Clarias gariepinus* Burchell) did not have an effect on Ht, Hb, white blood cells, red blood cells, MCV, MCH, MCHC, and leukocytes differential counts, meaning that BSF meal did not compromise O_2 transportation to vital organs, body capability of defense against invading pathogens, and fish health status (Fawole et al., 2020). Although with no effect on Ht, a higher Hb concentration was recorded in Nile tilapia (*Oreochromis niloticus* L.) fed with 25% to 100% dietary maggot meal (*Musca domestica* L.) (Ogunji et al., 2008). A 35% dietary inclusion of cricket (*Gryllus bimaculatus* De Geer) meal had positive effects on African catfish levels of Ht, Hb, MCH and MCHC, thus indicating a higher health status in this group compared to fish fed the fish meal-based diet (Taufek et al., 2016).

Survival rate after pathogens challenge is usually used to measure fish disease resistance. Although scarcely assessed, studies pointed that dietary insect meals have the capability of improving fish disease resistance against common pathogens (Ming et al., 2013; Ido et al., 2015, 2019; Su et al., 2017; Song et al., 2018; Chaklader et al., 2019; Li et al., 2019; Stenberg et al., 2019). Barramundi fed a 45% poultry by product diet supplemented with 10% BSF meal showed higher survival rate against *Vibrio harveyi* than fish fed the fish meal-based diet (Chaklader et al., 2019). However, a reference diet and a diet with 60% of BSF meal inclusion lead to similar plasma antibody titers after vaccination against infectious pancreatic necrosis in Atlantic salmon (Li et al., 2019). Also in Atlantic salmon, dietary inclusion of 10% and 15% BSF meal, prior to isolation of head kidney cells, had no effect on the transcription of inflammatory-related genes during a viral (polyinosinic acid:polycytidylic acid, poly I:C) or a bacterial (lipopolysaccharide, LPS) challenge (Stenberg et al., 2019). A 2.5% inclusion of house fly maggot meal in diets for black carp (*Mylopharyngodon piceus* (Richardson)), and a 5% inclusion in diets for red sea bream (*Pargus major* (Temminck and Schlegel)), were effective in increasing fish resistance against *Aeromonas hydrophila* and *Edwardsiella tarda*, respectively (Ming et al., 2013; Ido et al., 2015). Survival rate of pearl gentian grouper (*Epinephelus lanceolatus* (Bloch)♂ x *Epinephelus fuscoguttatus* (Forsskål)♀), red sea bream, and yellow catfish (*Pelteobagrus fulvidraco* (Richardson)) against *V. harveyi*, *E. tarda*, and *E. ictaluri*, respectively was improved in fish fed diets with 7.5%, 10% and 27% of *T. molitor* meal (Su et al., 2017; Song et al., 2018; Ido et al., 2019).

Lysozyme and the complement system are markers of the nonspecific immune response being used as main indicators of innate immunity in fish. Lysozyme is a bactericidal enzyme that lysis the constituents of the peptidoglycan layer of Gram-positive bacteria cell walls. Besides bacteriolysis, lysozyme also promotes phagocytosis by directly activating phagocytes cells or indirectly due to its opsonic nature (Saurabh and Sahoo, 2008). Complement is a system of serum proteins responsible for several immune functions such as elimination of invading pathogens and altered cells, phagocytes recruitment, promotion of inflammatory responses, and modulation of adaptive immune responses (Nakao et al., 2011). In yellow catfish, diets with graduate levels of *T. molitor* meal (9%, 18% and 27%) led to higher plasma lysozyme activity 24 hours after a bacterial challenge with *E. ictaluri*, compared with fish fed a fish meal-based diet (Su et al., 2017). Similarly, an enhanced serum lysozyme activity was observed in mandarin fish (*Siniperca scherzeri* Steindachner) fed with 30% *T. molitor* meal dietary inclusion level (Sankian et al., 2018). However, in European sea bass and rainbow trout serum lysozyme activity was not affected by the dietary inclusion of 25% *T. molitor* meal (Henry et al., 2018a, b). Additionally, in European sea bass a reduction of serum ceruloplasmin and myeloperoxidase activities and nitric oxide concentration, denoted an anti-inflammatory effect of dietary *T. molitor* meal, whereas an increase in serum trypsin inhibition activity indicates an immunostimulant activity (Henry et al., 2018b). Higher serum myeloperoxidase and trypsin inhibition activities were observed in rainbow trout fed a 50% *T. molitor* diet, together with a faster serum activity against *Escherichia coli* (Henry et al., 2018a). In barramundi, a 45% poultry by product diet supplemented with 10% BSF led to higher serum lysozyme and bactericidal activities, and higher relative expression of complement components 3 and 4, in fish challenged with *V. harveyi* compared with the same group before challenged (Chaklader et al., 2019). A 2.5% inclusion of maggot meal in diets for black carp was enough to improve serum lysozyme and complement components 3 and 4 (Ming et al., 2013), whereas in red sea bream even a low level of maggot meal (0.75%) enhanced peritoneal leukocyte phagocytic activity (Ido et al., 2015).

Besides the positive effects on fish nonspecific immune response, insect meals can also enhance components of the adaptive immune system and other immune elements such as proinflammatory cytokines. Thus, in yellow catfish, 9% and 18% *T. molitor* meal dietary inclusions increased the liver and spleen transcription levels of the immunoglobulin M (IgM), the predominant antibody type in fish, whereas IgM plasma levels were enhanced at 18% and 27% *T. molitor* meal dietary inclusion levels (Su et al., 2017). Additionally, an up-regulation of the gene expression of the major histocompatibility complex, which plays a key role in transporting peptide antigens to T cells, was observed in the spleen and kidney of yellow catfish fed a diet with 18% *T. molitor* meal inclusion before and after an *E. ictaluri* challenge, respectively (Su et al., 2017). In contrast, feeding barramundi with a fish meal and a 10% BSF meal supplemented diets

had no effect on head kidney MHC class II gene expression, both before and after challenge groups with *V. harveyi* (Chaklader et al., 2019). The gene expression of the proinflammatory cytokine IL-1β, a commonly used immune-regulatory gene in fish, was up-regulated in the liver and spleen of fish fed a diet with 9% and 18% *T. molitor*, respectively, before and after bacterial challenge (Su et al., 2017). In Atlantic salmon, a 60% BSF meal dietary inclusion level, increased both proximal and distal intestine transcription levels of cd3γδ and foxp3, meaning that BSF meal seemed capable to stimulate regulatory T cell activity in these intestinal segments (Li et al., 2019).

The enhanced immune function and improved resistance against pathogens reported in fish fed with dietary insect meals might be related to insects' immune-activating polysaccharides such as chitin, silkrose, or dipterose (Esteban et al., 2000, 2001; Cuesta et al., 2003; Gopalakannan and Arul, 2006; Harikrishnan et al., 2012; Ohta et al., 2014, 2016; Shanthi Mari et al., 2014;), and/or with the presence of antimicrobial peptides (Hou et al., 2007; Fu et al., 2009; Elhag et al., 2017). Crustacean chitin provided by intraperitoneal injection, incubated with head-kidney lucocytes or included in the diet, had an immunostimulatory effect on both innate humoral and cellular immune response of gilthead sea bream (Esteban et al., 2000, 2001; Cuesta et al., 2003). Dietary chitin and the chitin derivative chitosan enhanced the innate immune system of grouper (*Epinephelus bruneus* Bloch), Jian carp and *Cirrhinus mrigala* Hamilton, thus conferring disease resistance against *V. alginolyticus*, *A. hydrophila and Aphanomyces invadans*, respectively (Gopalakannan and Arul, 2006; Harikrishnan et al., 2012; Shanthi Mari et al., 2014). The mechanisms by which chitin modulates the immune system are by acting as a pathogen-associated molecular pattern and binding to pattern recognition receptors inducing cytokine and chemokine production (Lee et al., 2008). Other immune-activating polysaccharides such as dipterose isolated from melon fly pupae [*Bactrocera cucurbitae* (Coquillett)] and silkrose isolated from silkmoth pupae (*Antheraea yamamai* Guérin-Méneville), were able to activate mammalians innate immune response against various pathogenic microorganisms and viral infections (Ohta et al., 2014, 2016). Insects also possess antimicrobial peptides that can exhibit activity against microbial diseases. Elhag et al. (2017) isolated seven new gene fragments of three types of antimicrobial peptides from BSF with inhibitory activity against Gram-positive and Gram-negative bacteria and fungus. Antifungal and antibacterial activity substances were also obtained from the hemolymph and extracts of the housefly (Hou et al., 2007; Fu et al., 2009).

Overall, insect meals seem capable of positively modulating fish immune response and disease resistance, which make them promising as feedstuffs to reduce antibiotic use in fish farming. However, the specific role of insects' immune-activating polysaccharides and/or antimicrobial peptides in promoting fish health needs to be further investigated.

16.3.3 Oxidative status

In fish, as in all aerobic organisms, reactive oxygen species (ROS) are highly reactive chemicals produced from oxygen at a controlled rate during normal cell metabolism (Halliwell and Gutteridge, 2015). However, under stressful conditions such as environmental and nutritional alterations, ROS production may increase leading to oxidative stress if the defense mechanisms are not effective in scavenging ROS (Martínez-Alvarez et al., 2005). The detrimental effects of oxidative stress include oxidative damage of molecules of great biological importance, including proteins, DNA, carbohydrates and lipids (Halliwell and Gutteridge, 2015). Lipid peroxidation is a process initiated by ROS that attacks lipids containing carbon-carbon double bound(s), especially PUFAs. In fish, and mainly in carnivorous species, particular attention must be given to this process as fish tissues and diets are rich in PUFA (Sargent et al., 2002). The overall effects of lipid peroxidation include the decrease of membrane fluidity, the increase in membrane permeability, and damage to membrane proteins, thus inactivating receptors, enzymes and ion channels (Halliwell and Gutteridge, 2015).

The first line of defense against ROS includes the key antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). SOD catalyzes the dismutation of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2), which is later broken down by catalase and/or GPx into molecular oxygen and water (Halliwell and Gutteridge, 2015). Additionally, glutathione reductase plays a crucial role in the modulation of GPx activity as it catalyzes the NADPH-dependent regeneration of reduced glutathione from the oxidized form generated by GPx (Halliwell and Gutteridge, 2015).

Changes in antioxidant capacity should be taken into consideration for evaluating the nutritive value of alternative protein sources for fish meals, as beyond growth performance, these feedstuffs may also influence the organism's health status. However, in comparison with plant feedstuffs effects, insect meals' effects on fish oxidative status have been seldom addressed (Table 16.6), with the majority of the studies focusing on freshwater species (Ogunji et al., 2011; Dong et al., 2013; Ming et al., 2013; Ji et al., 2015; Taufek et al., 2016; Li et al., 2017; Su et al., 2017; Elia et al., 2018;

TABLE 16.6 Insect meal effects on fish oxidative status.

Fish species/weight (g)/days of feeding	Insect species	Insect form	% Insect meal inclusion	% FM substitution	Results	References
African catfish (*Clarias gariepinus*)/≈ 4/60	Partially defatted *Hermetia illucens*	Larvae meal	6, 11.5, and 17	25, 50, and 75	→serum MDA, serum CAT (6%) ↗serum CAT (11.5% and 17%) ↘serum SOD	Fawole et al. (2020)
African catfish (*Clarias gariepinus*)/≈ 13/49	Full fat *Gryllus bimaculatus*	Adult meal	26 and 35	75 and 100	→liver SOD and GST; CAT (26%) ↗liver CAT (35%)	Taufek et al. (2016)
Darkbarbel catfish (*Pelteobagrus vachelli*)/≈11/42	Full fat *Musca domestica*	Larvae meal	39	-	↘liver SOD, CAT, GPx, GR, T-AOC and GSH ↗liver ϒ-GT →intestine GSH ↗intestine T-AOC ↘intestine SOD, CAT, GPx, GR and ϒ-GT	Dong et al. (2013)
Gibel carp (*Carassius auratus gibelio*)/≈12/42	Full fat *Musca domestica*	Larvae meal	39	-	→liver CAT ↗liver SOD, GPx, GR, ϒ-GT, T-AOT and GSH →intestine SOD and CAT ↗intestine GPx, GR ϒ-GT and T-AOT ↘intestine GSH	Dong et al. (2013)
Japanese seabass (*Lateolabrax japonicus*) ≈14/56	Defatted *Hermetia illucens*	Larvae meal	5, 10, 14, and 19	16, 32, 48, and 64	→serum SOD, CAT and GPx ↘serum MDA	Wang et al. (2019)
Jian carp (*Cyprinus carpio* var. Jian) ≈10/56	*Hermetia illucens*	Larvae meal	3.5, 7, 10.5, and 14	25, 50, 75, and 100	→ serum MDA; serum T-AOC (3.5%, 7% and 10.5%) ↘serum T-AOC (14%)	Zhou et al. (2018)
Jian carp (*Cyprinus carpio* var. Jian)/≈35/59	Deffated *Hermetia illucens*	Larvae meal	3, 5, 8, and 11	25, 50, 75, and 100	→serum SOD and MDA; serum CAT (3% and 5%) ↗serum CAT (8% and 11%)	Li et al. (2017)
Jian carp (*Cyprinus carpio* var. Jian) ≈ 16/56	Full fat silkworm *Bombyx mori*	Pupae meal	6, 7, 8, and 9	50, 60, 70, and 80	→liver SOD and MDA (6%, 7% and 8%) ↘liver SOD (9%) ↗liver MDA (9%)	Ji et al. (2015)
Mandarin fish (*Siniperca scherzeri*)/≈ 21/56	Full fat *Tenebrio molitor*	Larvae meal	10, 20, and 30	10, 20, and 30	→serum SOD; GPx (10% and 20%) ↗serum GPx (30%)	Sankian et al. (2018)
Pearl gentian grouper (*Epinephelus lanceolatus* ♂ x *Epinephelus fuscoguttatus* ♀)/≈7/50	Deffated *Tenebrio molitor*	-	2.5, 5, 7.5, 10, and 12.5	6, 12, 18, 25, and 31	→liver GR (5%, 7.5%, 10% and 12.5%); SOD (2.5%, 5%, 10% and 12.5%); MDA (2.5% and 7.5%) ↗liver GR (2.5%); MDA (5%, 10% and 12.5%) ↘liver SOD (7.5%)	Song et al. (2018)

(*Continued*)

TABLE 16.6 (Continued)

Fish species/weight (g)/days of feeding	Insect species	Insect form	% Insect meal inclusion	% FM substitution	Results	References
Rainbow trout (*Oncorhynchus mykiss*)/≈116/90	Full fat *Tenebrio molitor*	Larvae meal	25 and 50	35 and 67	→pyloric caeca SOD, GR, G6PD and MDA ↗pyloric caeca GPx ↗proximal intestine SOD, CAT, GPx, G6PD and GR ↘proximal intestine MDA →distal intestine SOD and CAT (50%) ↗distal intestine GR and G6PD; SOD and CAT (25%) ↘distal intestine MDA	Henry et al. (2018a)
Rainbow trout (*Oncorhynchus mykiss*) ≈ 179/78	Partially defatted *Hermetia illucens*	Larvae meal	20 and 40	25 and 50	→liver MDA, SOD, CAT, totGPx, GST and GR; SeGPX, EROD and GSH + 2GSSG (20%) ↗liver EROD and GSH + 2GSSG (40%) ↘liver SeGPx (40%) →kidney MDA, SOD, CAT, totGPx, EROD, GR and GSH + 2GSSG ↗kidney GST (40%) ↘kidney SeGPx	Elia et al. (2018)
Siberian sturgeon (*Acipenser baerii*)/≈24/118	Highly deffated *Hermetia illucens*	Larvae meal	18.5 and 37.5	25 and 50	→liver MDA, CAT, EROD and GST; SOD, GPX and GR (18.5%) ↗liver SOD and GR (37.5%) ↘liver GPx (37.5%) →kidney MDA, CAT and GPx; GST (18.5%) ↗kidney EROD and GR; SOD and GST (37.5%)	Caimi et al. (2020b)
Yellow catfish (*Pelteobagrus fulvidraco*)/≈10/35	*Tenebrio molitor*	-	9, 18 and 27	25, 50 and 75	↘plasma MDA; SOD (18%) ↗plasma SOD (9% and 27%)	Su et al. (2017)

Symbols represent no effect (→), an increase (↗) or decrease (↘) in the response parameter of the insect meal diet relative to the fish meal control. In the case of insect meal being tested at more than one level, and when not indicated, it means that all levels had the same effect.
CAT, catalase; EROD, ethoxyresorufin O-deethylase; *FM*, fish meal; *GPx*, glutathione peroxidase; *SeGPx*, glutathione peroxidase selenium-dependent; *totGPx*, total glutathione peroxidase; *G6PD*, glucose-6-phosphate dehydrogenase; *GR*, glutathione reductase; *GSH*, reduced glutathione; *GSH + 2GSSG*, total glutathione; *GST*, glutathione s-transferase; *ϒ-GT*, ϒ glutamyl transpeptidase; *MDA*, malondialdehyde; *SOD*, superoxide dismutase; *T-AOC*, total antioxidative capacity.

Henry et al., 2018a; Zhou et al., 2018; Caimi et al., 2020a; Fawole et al., 2020), and only three in marine species (Sankian et al., 2018; Song et al., 2018; Wang et al., 2019).

Overall, insect meals were reported to have positive effects on fish oxidative status, by increasing antioxidant potential or decreasing oxidative damage (Table 16.6). Jian carp and African catfish serum catalase activities were enhanced in fish fed diets including BSF meal at 11.5% and 17%, respectively (Li et al., 2017; Fawole et al., 2020). In Siberian sturgeon, a 37.5% dietary inclusion of BSF meal led to an increase in liver and kidney SOD and glutathione reductase activities (Caimi et al., 2020a). Higher plasma SOD and serum GPx activities were recorded in yellow catfish and mandarin fish, respectively fed diets with 30% *T. molitor* meal (Su et al., 2017; Sankian et al., 2018). A 25% and a 50% *T. molitor* meal dietary inclusion also enhanced SOD, catalase, GPx, and glutathione reductase activities in the proximal

and distal intestine of rainbow trout (Henry et al., 2018a). Liver SOD and catalase activities in black carp, liver SOD, GPx, and glutathione reductase activities in gibel carp (*Carassius auratus gibelio* Bloch), and liver and gills catalase activities in common carp were all increased by dietary maggot meal levels of 2.5%, 39%, and 45%, respectively (Ogunji et al., 2011; Dong et al., 2013; Ming et al., 2013). A 35% dietary inclusion of field crickets improved catalase activity in African catfish liver (Taufek et al., 2016). An increase in total antioxidant capacity was observed in the liver and intestine of gibel carp fed with a 39% dietary house fly maggot meal (Dong et al., 2013).

Besides increasing fish antioxidant potential, insect meals also have the capability of diminishing oxidative damage, by decreasing lipid peroxidation specific markers such as malondialdehyde. Such an effect was observed in the serum of Japanese sea bass (*Lateolabrax japonicus*) fed diets with BSF meal up to 19% (Wang et al., 2019), in the plasma of yellow catfish, and in the proximal and distal intestine of rainbow trout fed diets with up to 27% and 50% *T. molitor* meal, respectively (Su et al., 2017; Henry et al., 2018a).

Although the majority of the works pointed out a positive effect of insect meals on fish oxidative status, there are few exceptions. Thus, 9% dietary silkworm meal in Jian carp and up to 12.5% *T. molitor* meal in pearl gentian grouper, led to a decrease in hepatic SOD activity and an increase of malondialdehyde content, thus denoting induced oxidative stress and lipid peroxidation (Ji et al., 2015; Song et al., 2018).

The antioxidant properties of insect meals can be associated with their chitin content and/or with the composition of the lipid fraction. *In vitro* and *in vivo* studies showed that chitin and their derivatives, such as chitosan were able to increase antioxidant enzyme activities and decrease lipid peroxidation levels (Ngo and Kim, 2014). In contrast, dietary chitin content was associated with lower GPx activity in both liver and kidney of Siberian sturgeon and rainbow trout fed BSF meal diets (Elia et al., 2018; Caimi et al., 2020a). Such an effect was associated with the capability of chitin to bind dietary inorganic (selenite and selenite) and organic (selenomethionine and selenocysteine) selenium (Se) forms (Pacini et al., 2012, 2013; Elia et al., 2018; Caimi et al., 2020a), thus making Se physiologically unavailable. Since the catalytic center of selenium glutathione peroxidase (SeGPx) contains selenocysteine, its unavailability due to the chitin bond may impair SeGPx activity. Besides chitin content, dietary lipid levels and composition can also interfere with antioxidant enzyme activities and peroxidation processes. The higher antioxidant enzymes activities observed in Siberian sturgeon fed isolipidic BSF-based diets could in part be explained by the higher levels of total fatty acids, saturated fatty acids and monounsaturated fatty acids, and the lower PUFA to saturated fatty acid ratio recorded in the 50% fish meal replacement level (Caimi et al., 2020a). As the susceptibility of fish tissues to suffer lipid peroxidation is highly dependent on the degree of unsaturation of dietary lipids, the lower malondialdehyde level recorded in African catfish fed with BSF meal-based diets was probably related to their lower content of PUFAs compared with the fish meal-control (Fawole et al., 2020).

In conclusion, the majority of the studies reported an absence of effect or even a positive influence of insect meals on fish oxidative status. As pointed out by Martínez-Alvarez et al. (2005) insect meal effects on fish antioxidant defenses seem to be dependent on feeding behavior and on nutritional factors. Thus, the same maggot meal diet fed to gibel carp (an omnivorous species) resulted in an enhancement of antioxidant capacity, whereas in darkbarbel catfish (*Pelteobagrus vachellii* (Richardson)) (a mixture of carnivorous and omnivorous species) led to a decrease (Dong et al., 2013). Nutritional factors such as dietary chitin and lipid contents, and lipid composition can also interfere with insect meals antioxidant capacity (Caimi et al., 2020a; Fawole et al., 2020).

16.3.4 Gut microbiota

The gut microbiota can be defined as a complex, dynamic biological system composed of commensal, symbiotic and pathogenic microbial communities, mainly consisting of bacteria, but also including archaea, fungi, protozoa, and viruses. The gut microbiota plays a central role in the host health maintenance, as it performs several metabolic functions, influences nutrient digestion and absorption, modulates the immune and digestive system development and protects the fish from environmental pollutants (Evariste et al., 2019). The development of the 16 S rRNA gene-based approach with a next-generation sequencing platform has recently allowed the characterization of the physiological gut microbiota in fish. Proteobacteria, Firmicutes, and Bacteroidetes are the dominant bacterial phyla, with Carnobacteriaceae, Leuconostocaceae, Aeromonadaceae and Vibrionaceae representing the most common families (Ikeda-Ohtsubo et al., 2018).

So far, only a few studies have investigated the impact of insect meal utilization on the gut microbiota of the main fish species (Table 16.7). Compared to fish meal-based diets, BSF meal inclusion up to 40% in diets for rainbow trout has been reported to increase the gut microbial α-diversity (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019) and richness (Bruni et al., 2018). A lower abundance of Proteobacteria and a higher

TABLE 16.7 Insect meal effects on fish gut microbiota.

Fish species	Insect species	Insect form	Feed composition	% Insect meal inclusion	% FM substitution	Days of feeding	Results	References
Rainbow trout (*Onchorynchus mykiss*)	Full-fat and defatted *Hermetia illucens*	Larva and prepupa meals	FM-based diet	30	30	35	↗α-diversity ↗Firmicutes and Bacteroidetes ↘Proteobacteria ↗Lactobacillaceae, *Corynebacterium* and *Bacillus*	Huyben et al. (2019)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Larva meal	FM-based diet	20 and 40	25 and 50	78	↗α-diversity and richness ↗Lactobacillaceae	Bruni et al. (2018)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Larva meal	FM-based diet	10, 20, and 30	10, 20, and 30	84	↗α-diversity ↗Firmicutes and Bacteroidetes ↘Proteobacteria ↗*Mycoplasma*	Rimoldi et al. (2019)
Rainbow trout (*Onchorynchus mykiss*)	Partially defatted *Hermetia illucens*	Prepupa meal	FM-based diet	10, 20, and 30	10, 20, and 30	84	↗α-diversity ↗Firmicutes and Bacteroidetes ↘Proteobacteria ↗Lactobacillaceae, *Actinomyces*, *Corynebacterium* and *Clostridium*	Terova et al. (2019)
Sea bream *(Sparus aurata)*, *Sea bass (Dicentrarchus labrax)*, Rainbow trout (*Onchorynchus mykiss*)	Full-fat *Tenebrio molitor*	Larva meal	FM-based diet	50 (sea bream and sea bass) and 60 (rainbow trout)	74 (sea bream), 71 (sea bass) and 86 (rainbow trout)	163 (sea bream), 70 (sea bass) and 90 (rainbow trout)	↗novel OTUs (62.2% in sea bream, 60% in sea bass and 33% in rainbow trout)	Antonopoulou et al. (2019)
Siberian sturgeon (*Acipenser baerii*)	Full-fat *Hermetia illucens* and *Tenebrio molitor*	Larva meal	FM-based diet	15	29 (HI) and 40 (TM)	60	↗*Clostridium leptum* subgroup and *Clostridium coccoides* — *Eubacterium rectale* cluster, *Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, Enterobacteriaceae and *Aeromonas* (HI) ↗*Clostridium coccoides* — *Eubacterium rectale* cluster, *Bacillus*, *Carnobacterium* and *Enterococcus* (TM)	Józefiak et al. (2019)
Zebrafish (*Danio rerio*)	Full-fat *Hermetia illucens*	Prepupa meal	FM-based diet	10.5 and 21	25 and 50	180	↗Enterobacteriaceae (HI reared on vegetable substrate) ↘Clostridia (HI reared on vegetable substrate)	Osimani et al. (2019)

Symbols represent an increase (↗), no effect (→) or decrease (↘) in the response parameter of the insect meal diet relative to the control. In the case of insect meal being tested at more than one level, and when not indicated, it means that all levels had the same effect. *HI*, *Hermetia illucens*; *TM*, *Tenebrio molitor*; *FM*, fish meal; *OTUs*, operational taxonomic units.

abundance of Firmicutes and Actinobacteria were also observed in BSF-fed fish (Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). These changes in phyla dominance resulted from an increased abundance of lactic acid bacteria [especially Lactobacillaceae (Bruni et al., 2018, 2019; Terova et al., 2019)], chitin-degrading bacteria [*Actinomyces* (Terova et al., 2019), *Corynebacterium* (Huyben et al., 2019; Terova et al., 2019), and *Bacillus* (Huyben et al., 2019)], butyrate-producing bacteria (*Clostridium* (Terova et al., 2019)), and symbiotic bacteria (*Mycoplasma* (Rimoldi et al., 2019)). Similar findings were also identified in juvenile Siberian sturgeon fed 15% of BSF meal (Józefiak et al., 2019), in terms of an increase in *Clostridium leptum* subgroup and *Clostridium coccoides—Eubacterium rectale* cluster (butyrate-producing bacteria), *Bacillus*, and *Carnobacterium*, *Enterococcus*, and *Lactobacillus* (lactic acid bacteria). Higher levels of Enterobacteriaceae (physiological in fish farmed near human populations) and *Aeromonas* (potential pathogenic bacteria) were reported in BSF-fed fish when compared to a 26% fish meal-based diet (Józefiak et al., 2019). The rearing substrate has also been reported to widely affect the gut microbiota of the BSF larvae, thus, in turn, influencing the gut microbiota of fish (Osimani et al., 2019). Increased abundance of Enterobacteriaceae—as well as lower Clostridia—were reported in zebrafish fed diets containing meal from BSF larvae reared on vegetable substrate when compared to larvae fed coffee by-product (Osimani et al., 2019). Less pronounced effects have been observed when using *T. molitor* meal instead of BSF in diets. Operational taxonomic units (OTUs) are a cluster of similar sequence variants of the 16S rDNA marker gene sequence used to categorize bacteria based on sequence similarity. Dietary *T. molitor* meal inclusion up to 60% resulted in the occurrence of the higher novel (OTUs) in gilthead sea bream (62.2% of all its OTUs) and European sea bass (60% of all its OTUs) in comparison with rainbow trout (33% of all its OTUs) (Antonopoulou et al., 2019). Differently, Józefiak et al. (2019) observed higher *Clostridium coccoides—Eubacterium rectale* cluster, *Bacillus*, *Carnobacterium* and *Enterococcus* genera only in juvenile Siberian sturgeon fed 15% of *T. molitor* meal than a 26% fish meal-based diet.

Overall, the above-mentioned findings suggest that insect meal inclusion in diets (especially BSF) can create a more diverse (and, in turn, stable) gut microbiota by selecting beneficial bacteria (lactic acid- and butyrate-producers) to the detriment of potential pathogens (Proteobacteria). These positive effects have been attributed to the chitin, which may act as a preferential substrate (prebiotic) for the lactic acid bacteria (Bruni et al., 2018), and show antimicrobial and bacteriostatic properties against several harmful Gram-negative bacteria (Rimoldi et al., 2019). The positive modulation of the gut microbiota, taken together with the overall preservation of both the morphology and the mucin dynamics, and the absence of significant histological alterations, suggests that the insect meal utilization does not negatively affect the gut health of the farmed fish.

16.4 Challenges and future perspectives

In the EU, insects kept for the production of feed are "farmed animals" (Reg. (EC) No 1069/2009). Therefore, they can only be fed using "feed grade materials" as listed in the catalog of raw materials (Reg. (EC) 2017/1017). This highly limits the potential and sustainability of the insect sector development, as products which could be used as animal or human feed/food have a higher environmental impact than low-value and unvalued organic side streams (Bosch et al., 2019; Smetana et al., 2019). A research priority in the EU, which could unleash the circular economy of the EU insect sector, is that required for certification of more diverse allowed substrates (IPIFF, 2019b). However, under no circumstance, the use of new substrates should compromise the safety of both animals and humans (Jülicher, 2020). Of primary importance is the guarantee of the safety of insect-based products. Therefore, it is crucial to deliver scientific data on the level of the chemical and microbiological hazards related to the utilization of these substrates.

The nutritional variability of the insect-derived products reflects the diverse origin of the insect diets and the production process, and it represents an issue as it could lead to divergent animal performance (Gasco et al., 2019a, b). Moreover, users (aquafeed producers) need consistent meal quality (CP and fat content, essential amino acids and fatty acid profile) as for fish meal or soybean meal. A solution to increase both meal quantity and consistency could be to collect insect meals from different producers, mix them and balance nutrients (additive supplementation). In this way, the market could be delivered with insect meals of constant quality.

Nowadays, insect-derived products are still too expensive, but the feed producers are willing to include these valuable raw materials in their formulation once the price of insect meals lowers down to a cost-effective price proportional to their protein content (Arru et al., 2019; Cadinu et al., 2020; Mancuso et al., 2019). This will be possible by improving the efficiency of the production technology and by scaling up the production process, which, in turn, could lead to prices that are more reasonable. There are already companies in EU countries (such as France: Ynsect, Innovafeed, Mutatec; Germany: Hermetia GmbH; Portugal: EntoGreen; The Netherland: Protix) or elsewhere in the world (Canada: Enterra; Malaysia: EntoFood; Tunisia: nextProtein; South Africa: Agriprotein; US: Enviroflight) that have

started large-scale production. Moreover, the worldwide increased demand for protein ingredients for feed purposes and the forecast increasing prices of conventional protein sources (fish and soybean meals) will decrease the gap in price between conventional and innovative protein sources (IPIFF, 2019a; Cadinu et al., 2020; Hong et al., 2020).

Chitin in insects is an indigestible fiber that could impair digestibility and be compared to an antinutritional factor. However, recent research highlighted that chitin at low levels is capable of stimulating the fish innate immune response and positively modulating the host microbiota. Moreover, it has been speculated that insects contain other bioactive compounds (antimicrobial peptides, particular fatty acids) that exert a positive effect on animal performance and health (Gasco et al., 2018a; Henry et al., 2018a, b; van Huis, 2020b). In an era where the pathogen's resistance to antimicrobials represents a major concern, the possible availability of natural compounds capable of controlling the negative effects of microbes is of great interest (Wu et al., 2018). This could be an opportunity as, if confirmed, these properties could help insect products to become more cost-effective.

16.5 Conclusions

Insect-derived products have a great potential to mitigate the shortage of conventional protein and lipid sources in aquafeeds. By using a correct balance of nutrients, the partial or total replacement of fish meal or fish oil has already been achieved in some fish species without compromising growth or health. For other species, on the other hand, the situation seems to be more complex, and it is not clear yet if this depends on nutrient imbalances in the diets used during the trials or on factors that are intrinsic to the insect meal included. Moreover, the current product availability, consistency and price result in negligible use of these products. The role of scientific research in conjunction with the insect production industry and legislators remains essential in order to allow the full development of the sector.

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Chapter 17

The role of insects for poultry feed: present and future perspective

Elizabeth A. Koutsos[1], Paul H. Patterson[2], Kimberly A. Livingston[3] and Tarra A. Freel[1]

[1]*EnviroFlight, LLC, Maysville, KY, United States,* [2]*Department of Animal Science, The Pennsylvania State University, University Park, PA, United States,* [3]*College of Veterinary Medicine, North Carolina State University, Raleigh, NC, United States*

17.1 Introduction

It is estimated that a third of all global meat consumption is poultry (Godfray et al., 2018). Presently most poultry species are fed diets that consist mainly of corn (or some other grain source such as wheat) and soybean meal. One-third of cereal grains produced in the world is fed to livestock, and an estimated 80% of all agricultural land is used for the production of feed or for grazing of livestock (Herrero et al., 2015; Mottet and Tempio, 2017). With the global population projected to reach 9.7 billion by 2050 (FAO, 2018) and demand for meat consumption increasing, more sustainable solutions for both feed and food production are needed. Insect-derived ingredients offer one solution to this growing need since insects have high concentrations of essential nutrients, generate high feed conversion to meat and eggs, and are capable of transforming waste biomass into valuable feed ingredients (Makkar et al., 2014). Insects require significantly fewer resource inputs, with some insect species having the capability to produce over 3000 times as much protein per acre compared to traditional agriculture species including plant protein and terrestrial animal protein sources (Koutsos et al., 2019). Recent life cycle assessment of commercial black soldier fly (BSF) (*Hermetia illucens* L.) farming systems demonstrates the reduced CO_2 and fossil resource depletion from this type of protein production system compared to that of wild-caught fish meal and vegetable oil production (PhI et al., 2020). Finally, many animal species naturally consume insects and thus it is logical to explore feeding formulations with greater proportions of insect-derived ingredients in their diet.

17.2 General nutrient composition of insects and insect-derived ingredients

Insect-derived ingredients include whole insects, partially defatted meal, and oil extracted from the whole insect [black soldier fly larvae (BSFL), meal and oil example; Fig. 17.1A–C]. Whole insects are generally dried but may also be fed live in some situations (Józefiak et al., 2016; Ipema et al., 2020; Star et al., 2020), Further processing may produce additional ingredients such as hydrolyzed protein sources, chitin and other nutritional and functional compounds. For any of these ingredients, it is critical to note that the management and feeding programs under which insects have been raised, as well as the method of processing, will result in the varied composition of the final ingredients. For example, the nutrient composition of BSF varies due to life stage (Fig. 17.2; Liu et al., 2017), as does nutrient digestibility (Do et al., 2020). In roosters, fat digestibility tended to be greater from older (days 23 and 29 posthatch) versus younger BSFL (days 14- and 18-days posthatch, $P < .10$). Similarly, digestibility of most amino acids was lower in roosters fed the youngest (days 0 and 11 posthatch) as compared to older aged BSFL (day 14–29, $P < .05$) (Do et al., 2020). Keeping this variability in mind, nutritionists and feed formulators should regularly evaluate nutrient composition to ensure appropriate inclusion in formulated feeds.

The nutrient composition of an assortment of insect species are detailed in previous publications (Bernard et al., 1997; Barker et al., 1998; Finke, 2002, 2013, 2015; Finke and Oonincx, 2017). This chapter focuses on a few common insect species reared at a commercial scale, including house crickets (*Acheta domesticus* L.), yellow mealworms (*Tenebrio molitor* L.), and BSFL, and for which nutrient composition of the whole insect is presented in Table 17.1.

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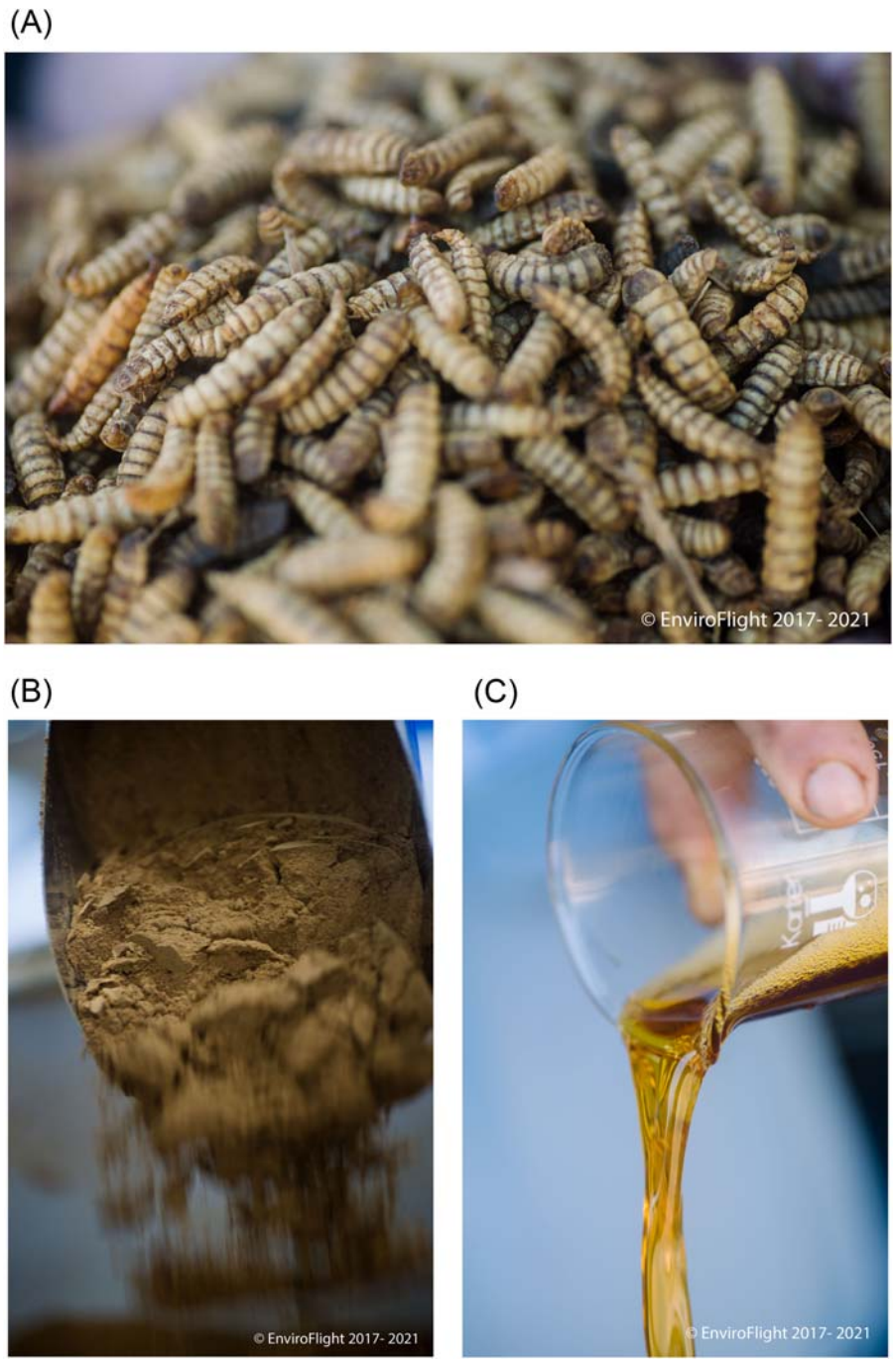

FIGURE 17.1 (A) Whole, dried black soldier fly (*Hermetia illucens*) larvae. (B) Partially defatted black soldier fly (*Hermetia illucens*) larvae meal. (C) Black soldier fly (*Hermetia illucens*) larvae oil.

The composition of partially defatted insect meal, used as a protein source in poultry diets, is shown in Table 17.2 and standardized to a 10% fat basis.

In general, insect-derived ingredients (whole dried insects and partially defatted meals) are an excellent source of protein and balanced amino acids for poultry production. Modern poultry diets have evolved beyond fulfilling simple crude protein requirements because commercial meat and egg birds today have a research-based requirement for essential amino acids to optimize not only performance but also bird health, welfare, efficiency and sustainability. While there are 11 essential amino acids that must be provided in the diet because the bird has no way to synthesize them for themselves, the first limiting amino acids are those in the shortest supply (Ravindran and Bryden, 1999; Vieira et al., 2004). Methionine is usually the first limiting amino acid in a commercial corn and soybean meal-based diet for broilers

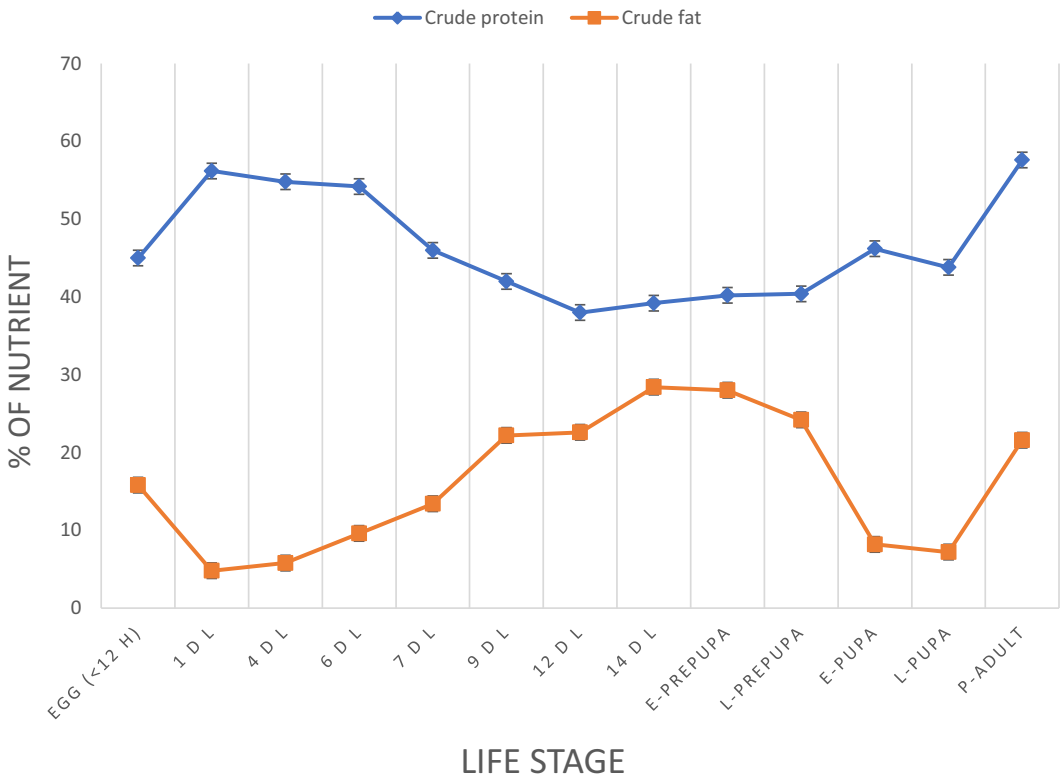

FIGURE 17.2 Variations in crude protein and crude fat content (dry matter basis, mean ± standard error, $n = 3$/life stage) in black soldier fly larvae and adults during different life stages (Liu et al., 2017).

TABLE 17.1 Nutrient composition of whole insects commonly reared at a commercial scale (Finke, 2013; Koutsos et al., 2019).

Nutrient (as is basis)	Yellow mealworm (*Tenebrio molitor*), larva	Black soldier fly (*Hermetia illucens*), larva	House cricket (*Acheta domesticus*), adult
Moisture (%)	61.9	61.2	69.2
Crude protein (%)	18.7	17.5	20.5
Crude fat (%)	13.4	14.0	6.8
Acid detergent fiber (%)	2.5	0.3	3.2
Ash (%)	0.9	3.5	1.1
Metabolizable energy (kcal/kg)[a]	2056	1994	1402

[a]*Predicted metabolizable energy using standard calculations [(gram of crude protein × 4.0) + (gram of crude fat × 9.0) + (gram of NFE × 4.0)].*

or layers because neither of these ingredients provides a concentrated methionine source, and vegetarian diets are more common today because of consumer preferences and organic stipulations. Corn typically contains 0.18% methionine and other traditional energy ingredients are not much higher in methionine, for example, barley and wheat contain 0.18% and 0.21%, respectively (NRC, 1994). Dietary soybean meal is enlisted for additional protein and methionine (0.67%), but combined with corn, provides less than the breeder recommendations for commercial production. For example, Hy-line recommends 0.34%−0.47% total methionine for W-36 white egg layers, depending on the age, phase of production and feed intake of the hen (Hy-Line, 2016), while a typical corn and soy diet (56% corn and 28% soy)

TABLE 17.2 Protein and amino acid composition of insect meals.

	Mealworm (*Tenebrio molitor*) larvae meal (*n* = 3)	Black soldier fly (*Hermetia illucens*) larvae meal (*n* = 4)	Cricket (*Acheta domesticus*) meal (n = 3)
Moisture (%)	5.20	3.59	5.20
Crude protein (%)	65.50	50.09	63.92
Arginine (%)	3.50	2.99	3.84
Histidine (%)	2.10	1.39	1.65
Isoleucine (%)	2.76	2.00	2.66
Leucine (%)	3.94	3.36	4.64
Lysine (%)	4.49	2.69	3.59
Methionine (%)	1.26	0.67	0.96
Phenylalanine (%)	2.35	1.96	1.40
Threonine (%)	2.31	1.38	2.25
Tryptophan (%)	NR[b]	0.60	0.39
Valine (%)	3.53	2.79	3.86
Alanine (%)	4.86	2.99	6.10
Cysteine (%)	1.56	0.42	
Tyrosine (%)	4.10	2.95	2.60
Crude Fat (%)	10.00	10.00	10.00

Note: All values are presented on a dry matter basis[a] and standardized to a 10% fat basis.
[a]*Values in table for insect meals obtained from De Marco et al. (2015); EnviroFlight internal data: Nakagaki et al. (1987).*
[b]NR = *not reported.*

would only provide approximately 0.29% methionine. Similarly, Aviagen recommends 0.47%−0.56% total methionine for Ross 708 broiler at a live weight target between 1.70 and 2.40 kg (Aviagen, 2014), while a diet consisting of 51% corn and 41% soy would only provide about 0.37% methionine. Insect meals including crickets (Nakagaki et al., 1987), BSFL (Schiavone et al., 2017b), and silkworms (Zhou and Han, 2006) can provide methionine levels ranging from 0.856% to 3.31% and closer to the levels found in meat meals (0.75%), poultry by-product meal (0.99%), and fish meals (1.63%) (NRC, 1994). In short, the greater concentration of protein and amino acids and the balance of essential amino acids provided by insect meals allow for dietary inclusions that can reduce or eliminate the need for synthetic methionine and other essential amino acid supplementation.

It is important to note that insect protein content may vary due to their diet; in general, higher protein rearing diets resulted in higher protein composition than when insects were fed lower protein diets in BSFL, yellow mealworms and Argentinean cockroaches (*Blaptica dubia* Serville) (Oonincx et al., 2015). Similarly, lipid content may vary significantly with insect diet and age (Liu et al., 2017). Insect lipid content does not necessarily reflect diet lipid content, i.e., higher fat diets do not necessarily produce higher fat insects, but insect fatty acids (as a percentage of total fatty acids) are more reflective of diet fatty acid proportions (Oonincx et al., 2015). In particular, work has focused on the enrichment of insects with long-chain omega-3 fatty acids. This has been accomplished in BSFL by feeding brown algae (*Ascophyllum nodosum*), which increased eicosapentaenoic acid (EPA) (20:5n-3) concentrations in the larvae (Liland et al., 2017), and by feeding fish offal which increased total omega-3 polyunsaturated fatty acid levels (Stvelssaturet al., 2007). Additionally, essential fatty acids including linoleic acid (C18:2) and α-linolenic acid (C18:3) may also be present in adequate quantities to meet the needs of poultry (El-Hack et al., 2020); but that is dependent on the insect feeding substrate composition and in the case of insect meal, the proportion of fat remaining in the product. Finally, some of

the fatty acids present in insect-derived ingredients, such as lauric acid, may have antimicrobial activity and thus influence gut function and overall animal performance (Skřivanová et al., 2006; Schiavone et al., 2017a), described later in more detail.

Mineral concentrations of insect-derived ingredients can vary due to species and diet substrate, most notably in Diptera (Dierenfeld and King, 2008; Finke, 2013; Tschirner and Simon, 2015; Spranghers et al., 2016). For example, BSFL accumulates calcium, sodium, and iodine from their substrate feed; BSFL meal can contain more calcium than fish meal and over an order of magnitude more calcium when compared to most other insect species (6.6%−9.3% Ca vs less than 1%, respectively, on a dry matter basis) (Finke, 2013; Makkar et al., 2014; Spranghers et al., 2016; Liland et al., 2017; Wang and Shelomi, 2017). The impact of diet on BSFL magnesium is unclear, with some data indicating that Mg content increases linearly with dietary Mg intake (naturally occurring Mg from seaweed; Liland et al., 2017), while other data suggest that rearing substrate and associated Mg level did not impact BSFL Mg concentration (Spranghers et al., 2016). Further work may be warranted to directly address this question in response to different sources and levels of dietary Mg.

It is not clear which trace minerals are essential for the growth of insects, however, several trace minerals considered essential for vertebrates appear to be regulated by insects; and thus, deposition is not directly responsive to diet. For example, copper and zinc deposition appear to be regulated in insects, whereas other minerals (e.g., selenium) may bioaccumulate in response to increasing dietary inclusion levels (Crawford et al., 1996; Maryanski et al., 2002; Vijver et al., 2003; Arnold et al., 2014; van der Fels-Klerx et al., 2018). Bioaccumulation of heavy metals in insects depends on the species and the metal in question, as well as the life stage of the insect (van der Fels-Klerx et al., 2018). For example, cadmium can accumulate in crickets and BSFL (Diener et al., 2011; Bednarska et al., 2015; Diener et al., 2015), and arsenic can accumulate in the yellow mealworm, BSFL and other insect species (van der Fels-Klerx et al., 2016; Biancarosa et al., 2018; van der Fels-Klerx et al., 2018). Though some insects may have the natural ability to rid their bodies of heavy metals via detoxification enzymes, it requires extra energy and therefore threatens the development and health of the insect (Calow, 1991; Stone et al., 2002; Diener et al., 2011). Thus, the risk of exposing insects to excessive heavy metals in feeds should be avoided and taken into consideration when selecting feedstocks, and by routine verification of the mineral content of both the feedstock and the insect-derived ingredient.

Water-soluble vitamins are generally in good supply in insects, with the exception of thiamin which may be destroyed via thiaminase activity postmortem (Nishimune et al., 2000). Fat-soluble vitamins are variable; vitamin A is not generally found in insects, although carotenoid pigments (xanthophylls and pro-vitamin A carotenoids) are generally in good concentration and are reflective of the diet that the insect consumed (Giovannucci and Stephenson, 1999; Finke, 2015). Vitamin D is variable depending on insect species, diet, and exposure to ultraviolet b (Oonincx et al., 2018). Vitamin E deposition in insects is generally diet-dependent (Finke, 2015).

The available energy content of insect-derived ingredients varies with insect species and fat content of the ingredient. True metabolizable energy, nitrogen corrected (TME_n) of BSFL, house cricket and mealworm meals for poultry ranged between 3990 and 5273 kcal/kg on a dry matter basis with mealworm meal having the highest TME_n ($\bar{x}$= 5199 kcal/kg dry mater (DM); crude protein: $\bar{x}$= 53.4% DM; crude fat: $\bar{x}$= 19.6% DM; $n = 3$) then BSFL meal (TME_n: $\bar{x}$= 4079 kcal/kg DM; crude protein: $\bar{x}$= 53.6% DM; crude fat: $\bar{x}$= 30.1% DM; n = 2) and cricket meal (TME_n = 4223 kcal/kg DM; crude protein = 67.4% DM; crude fat = 19.4% DM; $n = 1$), respectively (Matin, 2019). TME_n for these three insect species are all higher than NRC reported values for soybean meal (dehulled, solvent extracted), corn, and fish meal (NRC, 1994).

17.2.1 Impacts of processing method and form

The nutrient composition of insects can vary depending on the processing method, and the physical form of the finished ingredient. For example, the nutritional composition of mealworms is altered by the drying method (Table 17.3) (Kröncke et al., 2019). Vacuum- and freeze-drying resulted in slightly lower protein content (dry matter basis) as compared to rack oven-drying (120°C for 1 hour), while vacuum-dried larvae had the highest lipid content, and both vacuum- and oven-dried larvae had slightly higher concentrations of secondary metabolites indicative of lipid oxidation, most likely due to the high-fat content of these larvae, in combination with heat exposure. Finally, in vitro zinc digestibility was lowest with oven-drying, suggesting that high-temperature heating may reduce trace mineral bioavailability slightly more than that of other drying methods. Microwave drying of BSFL was shown to reduce the predicted amino acid digestibility as compared to conventional drying and resulted in a slightly different amino acid profile (Table 17.4) (Huang et al., 2019). Using scanning electron microscopy, the authors demonstrate that the proteins generated from these two drying methods had different physical characteristics, including more rigid, compact particles from

TABLE 17.3 Nutritional composition of mealworm larvae (*Tenebrio molitor*) before and after drying (Kröncke et al., 2019).

Parameter	Before drying	Rack oven dried	Vacuum dried	Freeze dried
Moisture (g/100 g)	62.87 ± 0.27^{a}	0.87 ± 0.03^{b}	1.70 ± 0.09^{c}	9.83 ± 0.03^{d}
Protein (g/100 g)	53.53 ± 0.28^{a}	56.30 ± 0.32^{b}	53.23 ± 0.20^{a}	$52.23 \pm 0.19^{a,c}$
Fat (g/100 g)	27.13 ± 0.03^{a}	27.27 ± 0.09^{a}	29.57 ± 0.02^{b}	26.80 ± 0.06^{c}
Fiber (g/100 g)	6.47 ± 0.09^{a}	7.10 ± 0.06^{b}	$6.83 \pm 0.03^{b,c}$	7.53 ± 0.09^{d}
Ash (g/100 g)	3.27 ± 0.12^{a}	3.43 ± 0.18^{a}	3.40 ± 0.15^{a}	3.43 ± 0.12^{a}
Myristic acid (C14:0)	NR[f]	2.61 ± 0.05^{a}	2.87 ± 0.05^{a}	2.20 ± 0.08^{b}
Palmitic acid (C16:0)	NR	18.08 ± 0.30^{a}	21.89 ± 0.76^{b}	$17.41 \pm 0.39^{a,c}$
Palmitoleic acid (C16:1)	NR	1.95 ± 0.01^{a}	1.57 ± 0.04^{b}	1.42 ± 0.02^{c}
Stearic acid (C18:0)	NR	2.70 ± 0.09^{a}	4.27 ± 0.26^{b}	3.52 ± 0.05^{b}
Oleic acid (C18:1)	NR	36.56 ± 0.35^{a}	32.93 ± 1.11^{a}	36.07 ± 0.74^{a}
Linoleic acid (C18:2)	NR	36.44 ± 0.63^{a}	34.99 ± 0.25^{a}	37.66 ± 1.12^{a}
Linolenic acid (C18:3)	NR	1.64 ± 0.06^{a}	1.48 ± 0.03^{a}	1.66 ± 0.10^{a}
P/S[e] ratio	NR	1.63 ± 0.06	1.80 ± 0.06	1.71 ± 0.09

Note: Nutrients presented on a dry matter basis, with mean +/− SEM ($n = 3$).
[a–d]Means within a row that do not share the same letters are significantly different ($P < .05$).
[e]*P/S = Polyunsaturated/saturated fatty acids.*
[f]*NR = not reported.*

microwave drying, which may be the basis for predicted reduced digestibility. It is important to note, however, that the method of protein extraction was chemical-based, followed by lyophilization, which is quite different from the traditional commercially used methods for protein extraction, which are generally mechanical in nature. Finally, heat-based drying methods, in general, reduce the concentration of both water- and fat-soluble vitamins as compared to live insects (Kinyuru et al., 2010).

Further processing of insects into partially defatted meal and oil will also change nutrient parameters. The degree of lipid removal from the insect during the preparation of the meal varies significantly. In one trial examining in vitro digestibility of yellow mealworm and BSFL meal obtained from a variety of sources, crude fat content ranged from 17% to 29% fat for mealworm meals and from 11% to −29% fat for BSFL meal sources (Marono et al., 2015). Additionally, the acid detergent fiber (ADF) fraction, a common indicator of chitin content, varied significantly within and between insect meal types, and ADF content is negatively correlated with in vitro protein digestibility. As the insect industry continues to develop methods for production, processing and further processing, it will be critical to have ongoing nutritional analyses to ensure that impacts on composition and digestibility are understood and able to be integrated into commercial diet formulation.

17.2.2 Functional aspects of insects in poultry diets

There is potential for insect-derived ingredients to provide functional properties in addition to essential nutrients and energy. Chitin, antimicrobial peptides (AMPs), and antimicrobial fatty acids are being evaluated for their ability to modulate and/or optimize performance of poultry under stressful or disease challenge situations.

17.2.2.1 Antimicrobial peptides

AMPs are secreted by insects as part of their humoral immune response (Park et al., 2014; Harlystiarini et al., 2019). For example, 53 genes coding for AMPs have, thus far, been identified in BSFL with 26 of them being coded for defensin AMPs, which are cysteine-rich cationic proteins largely expressed by epithelial cells or neutrophils (Oppenheim et al., 2003; Vogel et al., 2018) and have activity against several types of potentially pathogenic bacteria including

TABLE 17.4 Amino acid composition (g/100 g of protein) of black soldier fly larvae proteins determined by two drying methods, followed by chemical extraction and lyophilization (Huang et al., 2019).

Amino acid	Oven dried	Microwave dried
Isoleucine (g/100 g)	5.08 ± 0.22[a]	5.34 ± 0.12
Leucine (g/100 g)	8.80 ± 0.12	8.50 ± 0.14
Lysine (g/100 g)	6.45 ± 0.15	7.03 ± 0.05
Methionine (g/100 g)	2.72 ± 0.02	2.74 ± 0.16
Cystine (g/100 g)	1.10 ± 0.35	0.66 ± 0.12
Total sulfur amino acids (g/100 g)	3.82 ± 0.25	3.40 ± 0.15
Tyrosine (g/100 g)	7.67 ± 0.12	7.51 ± 0.13
Phenylalanine (g/100 g)	6.24 ± 0.20	6.04 ± 0.15
Total aromatic amino acids (g/100 g)	13.91 ± 0.15	13.56 ± 0.15
Threonine (g/100 g)	4.48 ± 0.16	4.28 ± 0.22
Valine (g/100 g)	5.80 ± 0.06	5.51 ± 0.15
Histidine (g/100 g)	4.11 ± 0.25	4.63 ± 0.05
Tryptophan (g/100 g)	1.32 ± 0.04	1.09 ± 0.02
Total amino acids (g/100 g)	40.89 ± 0.22	40.54 ± 0.18
Aspartic acid (g/100 g)	11.01 ± 0.03	10.90 ± 0.12
Glutamic acid (g/100 g)	10.82 ± 0.06	12.65 ± 0.15
Serine (g/100 g)	4.07 ± 0.15	3.69 ± 0.06
Glycine (g/100 g)	4.49 ± 0.23	4.12 ± 0.14
Arginine (g/100 g)	5.22 ± 0.15	5.64 ± 0.12
Alanine (g/100 g)	5.85 ± 0.22	5.24 ± 0.20
Proline (g/100 g)	4.79 ± 0.18	4.43 ± 0.15
Total nonessential amino acids (g/100 g)	59.11 ± 0.16	59.46 ± 0.18

[a]*Data presented as mean value ± standard deviation (n = 3).*

Helicobacter pylori, *Escherichia coli*, *Salmonella* spp., and methicillin-resistant *Staphylococcus aureus* (MRSA) (Park et al., 2014; Alvarez et al., 2019; Harlystiarini et al., 2019). Similarly, mealworms have demonstrated AMP activity, which could be induced by *S. aureus* challenge (Dobson et al., 2012) or by *B. bassiana* challenge (Yang et al., 2018), as has also been demonstrated in houseflies (*Musca domestica* L.) (Andoh et al., 2018) and BSFL (Vogel et al., 2018). As a consequence, insect-derived ingredients containing AMPs have the potential to improve the health and welfare of poultry and other livestock species that consume them. For example, broiler chicks fed 1%−3% BSFL (dried, whole larvae) had higher body weight gains and improved survival to a live *Salmonella gallinarum* challenge, as compared to chicks fed no BSFL. Thus, feeding insect-derived ingredients may provide a natural alternative to feed additives such as antibiotic growth promoters. AMPs appear to survive and maintain activity through the heating and processing of larvae and some AMPs have been shown to be thermally stable (Hao et al., 2008; Li et al., 2017). Further research is needed to fully understand the type and activity of AMPs within various insect species and their associated ingredients, and the application rate at which healthful benefits may be achieved under commercial conditions (Dörper et al., 2020).

17.2.2.2 Chitin

The exoskeleton of many insects is comprised primarily of chitin (Rinaudo, 2006) serving a structural role for the organism's body and protection from its environment (Lee et al., 2008). The chitin component of the exoskeleton is

covalently cross-linked to other cuticle proteins (termed "sclerotized protein"). The cuticle protein content and it is associated with crosslinking with chitin varies with species and life stage, and thus the total chitin content may be variable within and between species (Hahn et al., 2018). Additionally, the analytical tools for measuring chitin are limited and the general method of analysis, using the ADF extraction protocol likely overestimates total chitin content (Finke, 2007; Marono et al., 2015). Estimates of chitin content from ADF extraction protocols and new methods (combining ADF and acid detergent lignin (ADL) extraction protocols) are quite variable. In BSFL (age/life stage not described) ADF-determined chitin ranged from approximately 10% to 17%, while ADF-ADL-determined chitin ranged from 7% to 9%. Similarly, in yellow mealworms (age/life stage not described), ADF-determined chitin ranged from 14% to 22%, while ADF-ADL-determined chitin ranged from 6% to 9% (Hahn et al., 2018). Clearly, more standardized protocols are needed to accurately assess chitin content.

For many species, chitin is considered an indigestible fiber since most animals lack the enzyme chitinase, which is necessary for the digestion of this compound (Shahidi and Abuzaytoun, 2005; Tabata et al., 2017). However, chickens have the gene encoding for chitinase within the proventriculus, implying that they should be able to digest chitin at least partially (Suzuki et al., 2002; Tabata et al., 2017) and have an evolutionary history associating them with insects as a foodstuff. Several studies have demonstrated that chitin and its derivatives have health-promoting characteristics when used in poultry diets (Dörper et al., 2020). The incorporation of chitosan oligosaccharides into the diet of broilers increased the weight of immunological organs (Xiaofeng et al., 2017) and increased serum IgM levels, one of the predominant blood antibodies in birds (Deng et al., 2008), and may act as a prebiotic for the animal's microbiome (Li et al., 2007; Dörper et al., 2020).

17.2.2.3 Lauric acid

Lauric acid (C12:0) is a medium-chain fatty acid (MCFA) that makes up 21%−49% of the lipid found in BSFL oil, depending on the feeding program (Li et al., 2016). Many MCFAs have antimicrobial activity and may be able to be used as health-promoting supplements in poultry (Skřivanová et al., 2006; Schiavone et al., 2017a). In vitro experiments on the effect of lauric acid on *Clostridium perfringens* suggest that MCFAs enter a bacterial cell and disrupt the cytoplasmic structure while leaving the membrane intact (Skřivanová et al., 2006). Gram-positive bacteria appear to be more susceptible to MCFA inhibitory and bactericidal effects than are gram-negative bacteria (Skřivanová et al., 2006; Kim and Rhee, 2013). *In vivo*, broiler chickens challenged with *C. perfringens* and supplemented with mixed MCFAs (mainly lauric acid) had significantly fewer cases of necrotic enteritis lesions in the small intestine, likely due to the MCFA's effects (Timbermont et al., 2010).

17.3 Insects in meat bird production

17.3.1 Broilers

The evaluation of insects for meat birds (i.e., those birds reared for meat including broiler chickens, turkeys, and game birds such as pheasants and quail) production is not new. Initial efforts evaluated cricket meal (Nakagaki et al., 1987) and housefly larvae (Hwangbo et al., 2009) on broiler performance and nutrient digestibility. However, at that time, commercial production and processing were not yet developed (Nakagaki et al., 1987). Despite these limitations, Finke et al. (1985) demonstrated that dried, ground Mormon crickets could successfully replace soybean meal in practical corn/soy broiler diets to eight weeks of age with no significant differences in weight gain, feed conversion ratio (i.e., the ratio of feed consumed to body weight gained), or taste of the meat when limiting amino acids (methionine and arginine) are accounted for (Finke et al., 1985). In recent years, increased feed costs coupled with consumer desire for a smaller environmental footprint motivated researchers to revisit the substitution of conventional protein sources with insect meals in poultry diets on growth performance, meat quality, and nutrient digestibility (De Marco et al., 2015; Józefiak and Engberg, 2015; Altmann et al., 2018).

Insect meal trials have consistently demonstrated that these ingredients can replace some of the conventional protein sources found in meat bird diets (Leiber et al., 2017; Khan et al., 2018; Benzertiha et al., 2020). However, if they are added as a straight replacement without being properly formulated into the diet (e.g., without consideration for amino acid content or digestibility), often growth performance suffers as the nutrient composition is not the same as the conventional counterpart (Neumann et al., 2017). Moreover, when a dose-response is conducted, there is a consistent quadratic effect whereby the growth performance peaks between 8% and 15% of the dietary inclusion and levels above that can negatively affect performance (Dabbou et al., 2018; Wall et al., 2019). This varies with the insect species and nutritional composition, where BSFL meal can be included up to 15% of a broiler diet with no adverse effects on early

performance (Wall et al., 2019), while yellow mealworm supplementation starts to negatively affect the feed conversion ratio at greater than 4% inclusion (Elahi et al., 2020). Although considerable work has examined insect meal inclusion in broiler diets, work examining insect oil applications is limited at this time.

17.3.2 Other meat birds

Feeding live BSFL to turkeys significantly increases feed intake and body weight gain (fed at 10% of the expected feed intake) (Veldkamp and Van Niekerk, 2019). BSFL oil can replace 100% of soybean oil in the diet of young turkey poult without reducing growth performance, nutrient digestibility, gastrointestinal tract morphology, or breast/thigh quality, and with the additional benefit of reducing potentially pathogenic bacteria and tumor necrosis factor-α concentration (Sypniewski et al., 2020). Using full-fat BSFL meal as a protein source for young turkey poults, replacing up to one-third of soybean meal, did not affect final body weight or body weight gain, but did cause a linear decrease in the feed conversion ratio (Jankowski et al., 2021). The full-fat meal also initiated antiinflammatory benefits by decreasing tumor necrosis factor-α and increasing interleukin 6 levels in the small intestine and the activity of certain microbial enzymes (e.g., β-glucosidase) and butyric acid concentration in the cecal digesta (Jankowski et al., 2021).

Similar to data in broilers and turkeys, game birds and waterfowl can effectively use insect-derived ingredients as a partial or total replacement for other dietary protein sources, generally without significant impact on performance (Gasco et al., 2019). Muscovy ducks (*Cairina moschata domestica* (L.) Donkin), fed BSFL meal had higher protein and lipid digestibility than those fed corn gluten meal control diets (Gariglio et al., 2019). Japanese quail (*Coturnix japonica* Temminck and Schlegel) have been successfully reared on BSFL meal, replacing soybean meal (Cullere et al., 2016; Cullere et al., 2019) and BSFL and yellow mealworm meal were successfully utilized in the diets of Barbary partridge (*Alectoris barbara* (Bonnaterre)) (Loponte et al., 2017). In the latter trial, partridge fed insect meal had lower gastrointestinal tract weight and higher body weights compared to birds fed soybean meal-based diets, supporting a general improvement in gastrointestinal tract function and absorption among the insect-fed birds.

The carcass of a game bird shows some changes in color after being fed insect meals (yellow index in the case of Barbary partridge fed mealworm meal and red index in the case of Japanese quail fed BSFL meal). Additionally, the fatty acid content of tissues changes in response to feeding BSFL meal: the content of lauric acid was generally higher in eggs from quail fed BSFL meal, while n3 and n6 polyunsaturated fatty acids were reduced, possibly because of the fatty acid profile of the BSFL (Cullere et al., 2019). In general, despite some noted changes in carcass appearance and composition, there is no change in the sensory characteristics of the carcass (Gasco et al., 2019).

17.4 Insects in egg layer production

In laying hens, BSFL has been the primary insect species studied for its potential as a feed ingredient to date. Studies have demonstrated the capacity of BSFL to provide the necessary nutrients for the intensity of egg production with commercial laying hens (Maurer et al., 2016; Marono et al., 2017; Bovera et al., 2018; Mwaniki et al., 2018, 2020). In 2016, Maurer and colleagues fed a BSFL meal to commercial Lohmann Leghorn hens for the first time replacing 50% and 100% of the soybean meal with 12% and 24% BSFL meal, respectively (Maurer et al., 2016). Their BSFL meal contained 95.9% DM, 59.0% CP, 11.0% fat and 3.09% and 0.98% lysine and methionine, respectively. There was no significant difference in feed intake, egg production, feed conversion rate, mortality or hen health compared to the hens fed the control diet. However, egg weight (3.7 g) and albumen weight (3.0 g) were numerically reduced in the 24% BSFL meal inclusion treatment compared to the controls ($P = .15$ and .06, respectively). Despite the short duration of the feeding trial (3 weeks), the authors acknowledge the potential of BSFL meal as an alternative to soybean protein for laying hens. Marono et al. (2017) fed a dark brown, low-moisture (2.2%), low-fat (4.61%), high-protein (61.3%) BSFL meal to Lohmann Brown hens (24−45 weeks of age) at 17% of the diet replacing soybean meal. Feed intake, body weight egg production, egg weight and mass were significantly reduced by the BSFL meal treatment diet compared to hens fed a control corn/soybean meal diet. While the analyzed nutrient concentrations of the BSFL meal were adequate and formulated to Lohmann management specifications, the dark brown color of the meal suggested heat damage from drying that may have affected nutrient and amino acid availability and hence impacted performance (Marono et al., 2017).

In a two-phases study, BSFL meal (56.1% CP, 6.84% fat and 97.5% DM) was fed to Shaver White hens from 19 to 27 weeks and 28 to 43 weeks of age in basal diets consisting of corn, soybean meal, and wheat (Mwaniki et al., 2018, 2020). In the first phase, hens were fed diets with 0%, 5.0%, and 7.5% BSFL meal. Feed intake and body weight were significantly greater for the hens fed the 7.5% diet compared to the control or 5.0% BSFL meal diets (Mwaniki et al.,

2018). Daily hen egg production, egg weight, and mass followed a quadratic response to the BSFL meal diets with 5% lower than either the control or 7.5% diets ($P < .01$, .021, $< .01$, respectively). There was no significant impact of BSFL diet treatment on egg weight or Haugh units; but yolk color, shell thickness and breaking strength were increased with greater levels of the BSFL meal compared to the controls ($P < .01$). Yolk color change, from a more pale yellow color to a darker yellow color, is likely due to carotenoid concentration in BSFL meal, indicative of carotenoids in the diet of the BSFL. In the second phase, BSFL meal was incorporated into the diets at 0%, 10%, and 15% for partial to complete substitution of soybean meal (18.8%, 5.24%, and 0%, respectively) (Mwaniki et al., 2020). From 28 to 43 weeks of age, diet treatments had no impact on feed intake, egg production or feed conversion rate; but body weight was increased, and egg weight was reduced with BSFL meal supplementation ($P = .042$ and .01, respectively). Yolk color intensity was increased with increasing BSFL meal percentages, but BSFL supplementation had no impact on Haugh units, shell thickness or breaking strength in the second phase ($P > .05$).

Recent research evaluated BSFL meal (46.3% CP, 11.9% fat and 96.9% DM) at 0%, 8%, 16%, and 24% inclusion, nearly replacing soybean meal at the highest level (Patterson et al., 2021a). There was no wheat in these simple corn-soybean based diets fed to Hy-Line W-36 hens from 51 to 55 weeks old. Feed intake and egg production were significantly reduced, and body weight, egg weight and feed conversion rate were negatively impacted at the highest level of BSFL meal inclusion; however, there was no difference between the control, 8% and 16% BSFL meal treatments. But egg quality was similarly enhanced by the BSFL meal with higher yolk color (more yellow pigmentation of yolk) and specific gravity ($P < .035$), especially among the 16% and 24% BSFL meal treatments (Table 17.5).

Dietary BSFL oil (98% DM, 97% fat, 15.3% linoleic acid and 7840 kcal/kg AME_n) was evaluated with Hy-Line W-36 laying hens from 43 to 47 weeks of age (Patterson et al., 2021a). Control and treatment hens were fed a similar corn and soybean meal-based diet with levels of 0%, 1.5%, 3%, and 4.5% BSFL oil replacing soybean oil. In this study there was no impact of the BSFL oil ($P > .05$) on feed intake, body weight, egg production, weight or feed conversion rate (105 g, 1678g, 89.6%, 62.3 g and 1.886 kg/kg, averages respectively). However, as previously seen in our BSFL meal study and by Mwaniki et al. (2018) and Mwaniki et al. (2020), a linear increase in egg yolk color (yellow coloration) occurred in response to greater BSFL oil inclusion (Table 17.6, $P < .001$). Also, BSFL oil at 4.5% significantly improved egg specific gravity compared to the 1.5% eggs, and the 3.0% and control eggs were intermediate. Also, albumen height and Haugh units showed a numerical trend toward greater quality with greater oil inclusions (Table 17.6, $P = .065$ and $P = .085$, respectively).

Finally, dried whole BSFL (92% DM, 34% CP and 34% fat) was fed to the same W-36 hens from 60 to 64 weeks of age in corn-soy based diets with four levels of BSFL inclusion (0%, 6%, 12%, and 18%) (Patterson et al., 2021b). Hen body weight (average 1737 g), egg production (average 85.02%), and egg weight (average 64.17 g) were not significantly impacted by the BSFL diets compared to the control ($P > .05$). However, feed intake and feed conversion rate were significantly reduced by greater dietary levels of larvae inclusion (Table 17.7, $P < .05$). Measures of egg quality including blood spots, meat spots and yolk color were not impacted by the treatment diets ($P > .05$), although egg

TABLE 17.5 Impact of dietary black soldier fly (*Hermetia illucens*) larvae (BSFL) meal on hen performance (Patterson et al., 2021a).

Trt	Body wt (g)	Feed intake (g/hen/d)	Egg prod (%)	Egg wt (g)	FCR kg feed/kg egg	Egg yolk color	Egg specific gravity
Control	1668[a]	98.56[a]	83.97[a]	63.01	1.871	9.05[b]	1.0782[a,b]
BSFL Meal 8.0%	1643[a,b]	97.41[a,b]	85.44[a]	61.73	1.835	9.13[a,b]	1.0763[b]
BSFL Meal 16.0%	1663[a]	97.25[a,b]	86.02[a]	61.70	1.848	9.51[a]	1.0784[ab]
BSFL Meal 24.0%	1603[b]	92.40[b]	77.01[b]	59.96	2.009	9.33[a,b]	1.0799[a]
SEM	18.10	1.504	1.795	0.95	0.0469	0.123	0.00076
P-value	0.0509	0.0412	0.0071	0.1889	0.0584	0.0371	0.0090

[a,b]Means within a column with no common superscripts differ significantly ($P < .05$).

TABLE 17.6 Impact of dietary black soldier fly (*Hermetia illucens*) larvae (BSFL) oil on hen performance (Patterson et al., 2021a).

Trt	Body wt (g)	Feed intake (g/hen/d)	Egg prod (%)	Egg wt (g)	FCR kg feed/kg egg	Egg yolk color	Egg specific gravity	Albumen ht (mm)	Haugh unit
Control	1666	104	88.51	61.58	1.919	7.37^{b}	$1.0777^{a,b}$	8.69	92.44
BSFL Oil 1.5%	1679	105	88.83	62.45	1.885	7.77^{a}	1.0765^{b}	8.99	93.74
BSFL Oil 3.0%	1680	105	90.93	62.63	1.848	7.93^{a}	$1.0778^{a,b}$	9.12	94.32
BSFL Oil 4.5%	1685	106	90.23	62.39	1.893	7.93^{a}	1.0795^{a}	9.27	95.29
SEM	16.88	1.65	0.86	0.68	0.0340	0.09	0.00059	0.16	0.79
P-value	0.8603	0.8022	0.1791	0.7051	0.5355	0.0001	0.0066	0.0649	0.0849

^{a,b}Means within a column with no common superscripts differ significantly ($P < .05$).

TABLE 17.7 Impact of dietary black soldier fly (*Hermetia illucens*) larvae (BSFL) on hen performance (Patterson et al., 2021b).

Trt	Feed intake (g/hen/day)	FCR kg feed/kg egg	Egg specific gravity	Albumen ht (mm)	Haugh unit
Control	108^{a}	2.079^{a}	1.073^{b}	8.55^{a}	91.28^{a}
BSFL-6%	106^{ab}	1.934^{ab}	1.075^{a}	8.05^{ab}	88.01^{ab}
BSFL-12%	101^{bc}	1.918^{ab}	1.075^{a}	7.82^{b}	86.53^{b}
BSFL-18%	100^{c}	1.825^{b}	$1.074^{a,b}$	$8.09^{a,b}$	$88.08^{a,b}$
SEM	1.61	0.0522	0.0005	0.16	1.02
P-value	0.0048	0.0209	0.0177	0.0257	0.0133

^{a,b}Means within a column with no common superscripts differ significantly ($P < 0.05$).

specific gravity was higher and albumen height and Haugh units were lower among the BSFL treatment eggs compared to the controls ($P < .05$). Egg yolk fatty acid profile was also impacted by the BSFL dietary treatments. Greater amounts of yolk C12:0, C14:0, C14:1n5, and C21:0 were obtained with increasing dietary levels of larvae inclusion ($P < .05$); however, there was no impact of the treatments on yolk C18:2, linoleic acid or C18:3n3, alpha-linolenic acid ($P > .05$).

17.5 Impact of insect-derived ingredients on behavior and welfare

The feeding of insects to poultry has been hypothesized to improve animal welfare since insects would be a natural part of a free-ranging poultry diet, and that of wild counterparts (Zuidhof et al., 2003). In broilers, to help offset the rapid growth rate combined with restricted space and lack of environmental stimulation under commercial conditions, live

BSFL can be used as an environmental and nutritional enrichment to increase the activity of the birds thereby improving leg health (Ipema et al., 2020). Feeding live BSFL to turkeys lowers the incidence of potentially problematic pecking behavior (Veldkamp and Van Niekerk, 2019). Similarly, in laying hens, feather pecking is a welfare challenge which can be improved by the inclusion of live BSFL in the diet. Feeding live BSFL can provide environmental enrichment that helps improve the feather quality of laying hens by encouraging natural foraging behavior thus decreasing aggression (Blokhuis and Wiepkema, 1998; Star et al., 2020). Applications of insect feeding programs to support animal welfare goals of commercial poultry producers should not be underestimated.

17.6 Barriers and hurdles for use of insects in poultry diets

As previously described, the insect production parameters, including feedstock, development time and the processing method can significantly impact the concentration and digestibility of nutrients from insect-derived ingredients. The commercial adaptation of insect-derived ingredients in poultry diets faces additional barriers and complexity. It has been reported that the biggest obstacles in commercial insect production are technical, financial, and regulatory barriers (Sogari et al., 2019).

As with other feed ingredients, standards must be met to ensure the safety of processed insect products (Rumpold and Schlüter, 2013). Safety and quality assurance can be similarly achieved and includes testing for microorganisms, regular sampling for wet chemistry analyses, and retaining samples for follow up analysis. Furthermore, implementing an effective kill step during insect processing will reduce the risk of contamination with microorganisms (Fernandez-Cassi et al., 2019). Guidelines and best practices are currently being developed and adopted to standardize processing methods to ensure safety and quality in North America and the European Union (EU).

Another challenge with producing novel insect ingredients to use in animal feed is the required investment in equipment and infrastructure in combination with the technology learning curve to optimize production and efficiency. As with any developing industry, costs of equipment development and refinement are high at the onset of commercialization, but it is expected that costs related to insect production will be reduced over time (de Jong and Nikolik, 2021).

Regulatory approvals must be achieved before insect-derived ingredients may be consistently, and legally, incorporated into animal feeds (Sogari et al., 2019). Laws surrounding insects and insect-derived ingredients for animal feed vary depending on the country. In the United States (US), BSFL (whole dried and partially defatted meal) was defined as a feed ingredient by the Association of American Feed Control Officials (AAFCO) for salmonids and poultry in 2019. Within this definition, the BSFL may only be raised on a feedstock composed solely of feed grade materials. To date, larvae of BSF are the only insect species with an AAFCO ingredient definition, although dried insects of any species can generally be used in wild bird food (AAFCO, 2021). AAFCO is not an enforcement agency; but rather a forum where industry and feed control officials can exchange information which AAFCO then uses to develop standards to promote uniformity of regulations between states and provide acceptance at the US national regulatory level, although individual state regulators have authority to register ingredients within their state.

An in-depth description of the insect-derived ingredient sourcing, it's processing, research findings and/or a literature review assessing the utility and safety of the ingredient as an animal feed (specific to animal species, purpose, life stage or all inclusive) must be reviewed by the Food and Drug Administration (FDA) Center for Veterinary Medicine, followed by AAFCO committee and full membership review before receiving an AAFCO official definition,. The trials required to document safety can be costly and lengthy often requiring either individual company financing or collaboration across cooperative ingredient producers and their intended customers. The process from initiation of research trials to formal ingredient definition can be expected to take a minimum of 24 months starting at the point FDA gives a positive recommendation.

In some countries, such as South Korea, the consumption of insects is considered customary and there are no regulations for the use of insects as food and feed (Sogari et al., 2019). However, in countries in the European Union, insect production is a fairly new practice and legislation regarding safety and application is still under review. Currently, in the EU, insect proteins from seven commercially raised species are approved including the BSF, housefly, yellow mealworm, lesser mealworm (*Alphitobius diaperinus* Panzer), house cricket, banded cricket (*Gryllodes sigillatus* Walker), and field cricket (*Gryllus assimilis* Fabricius). The insects themselves may only be fed materials of plant origin with the exception of certain products of insect/animal origin which include milk, eggs, honey, and rendered fat or collagen from nonruminant animals. The use of insect species for aquaculture feed is allowed, as is an application for pets; but currently, poultry and swine may not be fed insects or insect meal as a component of a complete feed (Lähteenmäki-Uutela et al., 2018). However, fat derived from insects may be fed to any species (Sogari et al., 2019). The producers

of insect meal (i.e. processed insect proteins) in the EU must also abide by specific processing methods which are defined by EU regulatory agencies.

For international trade, it is important to have a thorough understanding of the laws of other countries as the importation of insect-derived ingredients generally requires the insects to be raised according to regulations specific to the country of import. They may require documents from the exporter's governing bodies certifying the practices in place at production facilities and attesting to the statements and guarantees made by or expected from the manufacturers. Additionally, registration and permit procedures vary by country. For example, in Canada the Canadian Food Inspection Agency (CFIA) requires a resident Canadian agent to authorize the registration application.

17.6.1 Organic classification

Certified organic poultry meat and egg production are growing worldwide. In the US there are currently 19.9 million certified organic laying hens representing almost 6% of the national flock, and the National Agricultural Statistics Service Organic Survey in 2019 reported some of the greatest increases (49 and 68%) are in the sale of broiler chickens and turkeys, respectively (USDA, 2019). Along with greater numbers of organic turkeys, broilers and layers are the challenge of a limited supply of certified organic ingredients. In the US approximately 75% of the corn and only 10% of the soybeans used are raised domestically and the rest must be imported (Einstein-Curtis, 2018). Furthermore, the rules in the US and Europe limit nonorganic ingredients in the diets of poultry and other livestock. In the US there are limits on synthetic methionine (2, 2.5, and 3 lbs/ton; 0.001, 0.00125, and 0.0015 g/kg) for layers, broilers and turkeys (USDA, 2020) that do not completely meet the requirements of high-performance flocks. Therein is the need for high-quality protein ingredients with a balanced complement of essential amino acids. As mentioned previously, high-protein insect meals with significant amounts of essential methionine and lysine could fill that void; however, current regulations for organic feedstuffs in both the US and EU require the use of organic inputs, which limits the potential production capacity and may increase the cost associated with insect production.

17.7 Summary and the conclusions

As the world's human population continues to grow, insects are gaining importance as animal feed due to their ability to efficiently turn waste into a high-quality source of nutrition with less impact on the environment than traditional livestock species. Poultry naturally consumes insects, and this system can be enhanced further by producing insects on a commercial scale for a more uniform and consistent nutritional composition. Insects are by their nature-rich in protein and a balanced source of essential amino acids. They are a good source of essential fatty acids linoleic and linolenic acids, and insects can be enriched with omega-3 fatty acids by their diet. Vitamins and minerals vary with the insect species and their diet, and BSFL can bioaccumulate calcium, manganese and other essential minerals. However, nonessential minerals can also concentrate in insect tissues and must be monitored. Beyond their nutritional profile, some of the fatty acids and peptides in insect-derived ingredients have antimicrobial activities. Lauric acid (C12:0) is believed to have activity influencing gut function and animal performance, and AMPs secreted by insects as part of their immune response have activity against several of the common poultry pathogens (*E. coli, Salmonella* and *Staphylococcus* species). Chitin, the exoskeleton structural component of insects, is largely considered indigestible for animals, although chickens have a chitinase enzyme in their proventriculus indicating they may partially utilize chitin. Research has shown chitin and its derivatives have some health-promoting properties, influencing immune organ and parameter function, increasing IgM levels, and acting like microbiome prebiotics. Whole insects and meals or extracted oils from insects can readily be incorporated into broiler and layer diets at 10% or more of the diet with excellent weight gain, feed conversion and production performance as well as maintaining meat and egg quality. Their continued cultivation and processing can contribute valuable nutrients for poultry and other livestock and are a sustainable alternative as world resources become increasingly limiting.

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Chapter 18

Insects as food for insectivores

Mark D. Finke[1,2] and Dennis Oonincx[1,2]

[1]*Mark Finke LLC, Rio Verde, AZ, United States,* [2]*Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, the Netherlands*

18.1 Introduction

Insects are generally considered a good source of most nutrients (DeFoliart, 1992) and many species have been analyzed for their nutrient composition. These analyses include insects taken from the field (Bukkens, 1997; Finke, 2015b; Gullan and Cranston, 2005; Punzo, 2003) and those commercially reared (Dierenfeld and King, 2008; Finke, 2002, 2013, 2015a; Oonincx et al., 2010; Oonincx and Dierenfeld, 2012; Ramos-Elorduy et al., 2002; Simpson and Raubenheimer, 2001). In nature, most insectivores consume insects and a variety of other arthropods including arachnids (scorpions, whipscorpion, ticks, and spiders), isopods, millipedes and centipedes. As a general rule, it is better for insectivores to be offered a varied diet rather than a single insect species as a mixed diet is less likely to result in nutrient deficiencies.

A number of variables can influence the chemical composition of insects, such as gender (Ali and Ewiess, 1977; Hoffmann, 1973; Sonmez and Gulel, 2008), stage of development (McClements et al., 2003), diet (Calvez, 1975; Oonincx and van der Poel, 2011; Ramos-Elorduy et al., 2002; Simpson and Raubenheimer, 2001) and environmental factors such as temperature (Hoffmann, 1973; Sonmez and Gulel, 2008), day length (Ali and Ewiess, 1977; Koc and Gulel, 2008; Shearer and Jones, 1996), light intensity and spectral composition (Oonincx et al., 2018) and humidity (Ali et al., 2011; Han et al., 2008; Nedvěd and Kalushkov, 2012).

Nutrient densities can be expressed based on the fresh or dry weight. As insectivores consume live prey, fresh weight is sometimes preferred. While water is a critical nutrient, in most cases providing it is not the primary function of offering insects as food. Furthermore, variation in moisture content strongly influences the nutrient density when expressed as fresh weight. This hampers a thorough nutritional comparison. Therefore, in this chapter nutrient densities are expressed on a dry matter basis. As indicated, large variations in dry matter content exist between species and developmental stages. As a rule of thumb, the dry matter content typically ranges from 13% to 42% of the fresh weight of a live insect (Barker et al., 1998; Bernard and Allen, 1997; Finke, 2002, 2013; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003).

18.2 Nutrient content of insects

18.2.1 Protein and amino acids

The protein content of insects is highly variable and ranges between 7.5% and 91% with many species containing around 60% protein on a dry matter basis (Barker et al., 1998; Bernard and Allen, 1997; Bukkens, 1997; Finke, 2002, 2013; Oonincx et al., 2010; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Oonincx et al., 2015; Punzo, 2003; Ramos-Elorduy et al., 2002). The protein content is commonly determined by multiplying the amount of nitrogen times 6.25, known as the crude protein content. This factor is underestimated if not all amino acids are properly quantified (Oonincx et al., 2019). Conversely, the presence of nonprotein nitrogen from compounds, such as chitin, uric acid, melanin and β-alanine, leads to overestimations of true protein content when using this factor. An alternative Kp of 4.76 for insects has been suggested based on amino acid data from only three species of insect larva analyzed by a single laboratory (Janssen et al., 2017). Other authors suggest Kp ranges of 4.21−5.05 and 5.25−5.33 for five insect species while reporting varying values for the same species and life stage (Belghit et al., 2019; Boulos et al., 2020). Recalculating the data from an even wider set of 20 insect samples, including 13 species and different developmental stages results in an average Kp of 5.81; ranging from 4.56 to 6.45 (Finke, 2002, 2007, 2013, 2015a, 2015b). This data confirms that a Kp of 6.25 is often a slight overestimate. However, until data for more species and at different life stages are accumulated, retaining a Kp of 6.25 is recommended to facilitate comparisons between studies.

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Amino acids are the building blocks of proteins. Certain amino acids are known as essential amino acids because they cannot be synthesized from simpler molecules by most animal species (Bender, 2002). It seems likely that the amino acid composition of insects (expressed as mg/g protein) is fairly constant within a given species and life stage. Wings, legs, mandibles, and other body parts have specific physical requirements in order to function properly; therefore, differences in amino acid composition between insects fed different diets are unlikely. Prepupae from black soldier fly larvae (*Hermetia illucens* L.) fed different diets had similar amino acid patterns (Lalander et al., 2019; Spranghers et al., 2017; Wang et al., 2020), as did tobacco hornworm larvae (*Manduca sexta* (L.)) fed two different diets (Landry et al., 1986). Additionally, a review of house cricket (*Acheta domesticus* L.) amino acid composition (expressed as mg of amino acids/g protein) from six different papers showed very similar amino acid patterns (Oonincx and Finke, 2021).

Prediction of an insect's amino acid profile based on species, age, and life stage is difficult at best. Table 18.1 shows the amino acid pattern of six commonly raised insect species and compares them to the amino acid requirements of rats, growing broiler chicks (poultry) and trout (NRC, 1994, 1995, 2011).

TABLE 18.1 Typical amino acids patterns (mg/g crude protein) of some common feeder insects and amino acid scores and first limiting amino acid for various species.

	Acheta domesticus	*Tenebrio molitor* larvae	*Zophobas mori* larvae	*Hermetia illucens* larvae/prepuape	*Blatta lateralis* nymphs	*Bombyx mori* larvae
Alanine	87.9	80.2	72.7	62.7	87.9	44.6
Arginine	65.7	60.0	57.4	52.8	79.3	65.6
Aspartic acid	79.1	81.0	83.1	88.3	73.9	41.4
Glutamic acid	109.2	112.1	127.0	103.7	118.8	99.5
Glycine	52.3	53.1	48.7	55.0	65.2	59.7
Histidine	22.8	30.2	31.1	32.4	28.9	25.8
Isoleucine	40.3	46.1	46.9	43.3	40.7	32.3
Leucine	78.6	84.9	80.4	69.9	63.0	52.7
Lysine	55.3	55.4	54.6	59.1	67.2	46.8
Methionine	15.8	13.3	12.2	18.8	17.6	13.4
Methionine + cystine	24.9	23.3	21.8	24.4	25.2	22.0
Phenylalanine	31.5	35.2	37.2	41.5	40.4	28.5
Phenylalanine + tyrosine	87.5	102.6	108.4	112.3	115.6	59.7
Proline	56.7	68.1	55.9	55.4	55.9	33.9
Serine	44.1	47.2	44.1	38.3	44.1	36.0
Threonine	35.7	40.3	39.9	39.0	41.5	30.6
Tryptophan	7.2	10.5	11.4	15.1	8.7	7.0
Valine	54.1	62.9	60.5	63.8	64.7	40.3
Taurine	6.1	0.4	ND	ND	ND	ND
Amino acids score/first limiting amino acid for						
Rats	38/ met + cys	36/met + cys	33/met + cys	37/met + cys	39/met + cys	34/met + cys
Poultry	64/ met + cys	60 met + cys	56/met + cys	62/met + cys	64/met + cys	56/met + cys
Trout	52/ met + cys	49/met + cys	46/met + cys	51/met + cys	53/met + cys	45/lys

The nutritional quality of insect protein has generally been described as good, but the quality depends on the digestibility of the amino acids and the match of the amino acid profile to the requirements of the insectivore in question (Finke et al., 1987, 1989; Ramos-Elorduy, 1997). Unfortunately, the amino acid requirements of most insectivores are unknown, so most comparisons have been made using more common laboratory animals, such as rats or chickens (Finke, 2002, 2013).

The first limiting amino acid depends on both the insect species being consumed and the species consuming the prey. Since different insectivore species likely require different proportions of amino acids, the first limiting amino acids could differ between species. In chickens fed purified diets where Mormon cricket (*Anabrus simplex* Haldeman) or house cricket meal was the sole source of protein, the first limiting amino acids were methionine and arginine (Finke et al., 1985; Nakagaki et al., 1987). When fed to growing rats, the first limiting amino acids in insect protein from yellow mealworm larvae (*Tenebrio molitor* L.), common housefly (*Musca domestica* L) larvae and adult Mormon crickets were methionine (Finke et al., 1987; Goulet et al., 1978; Onifade et al., 2001). Similarly, methionine and cystine were calculated to be the first limiting amino acids for rats in house crickets, yellow mealworm larvae, lessor mealworms (*Alphitobius diaperinus* (Panzer)), superworm larvae (*Zophobas morio* F.), larvae of the greater wax moth (*Galleria mellonella* L.), domesticated silkworm larvae (*Bombyx mori* L.), honey bee larvae and pupae (*Apis mellifera* L.), adult common houseflies, black soldier fly larvae, Turkestan roaches (*Blatta lateralis* Walker) and butter worm larvae (*Chilecomadia moorei* Silva) (Finke, 2002, 2013; Jensen et al., 2019; Poelaert et al., 2018). As such it seems likely that for most mammalian insectivores the sulfur amino acids are the first-limiting. For fish, birds and other species, especially those without a functioning urea cycle, other amino acids such as arginine, may be important.

18.2.2 Fats and fatty acids

The most common way to estimate insect fat content is by determining the total weight of all fat-soluble molecules (mostly lipids but also waxes and some other compounds). Fat tissue is used for energy storage in the body and is either obtained directly from the diet or synthesized from carbohydrates (Bender, 2002; Fast, 1970; Hanson et al., 1983). The main storage site for insect lipids is the fat body (Beenakkers et al., 1985). As a dietary component, fat is not only an important energy source but may also play a role in the palatability of the insect when fed to insectivores. Large variations in the lipid content (4.6%−64% dry matter) of insects have been reported (Barker et al., 1998; Bukkens, 1997; Finke, 2002, 2013; Punzo, 2003; Yang et al., 2006).

The behavioral ecology of a species likely influences the amount of fat stored (Thompson, 1973). In some species, males have a higher fat content than females (Fast, 1970; Nakasone and Ito, 1967). For species where male combat is customary (for instance Odonata), this can be explained by a need for greater energy reserves. In silkworms and certain Saturnids (Lepidoptera), males also have greater fat reserves than females (Beenakkers et al., 1985). Generally, however, females have greater fat reserves than males (Kulma et al., 2019; Lease and Wolf, 2011; Nestel et al., 2005; Zhou et al., 1995). Species that accumulate energy reserves for reproduction during their larval stages would have an increased fecundity potential, as eggs have a high lipid content (Beenakkers et al., 1985; Downer and Matthews, 1976; Lease and Wolf, 2011). Before oviposition, these females would have a higher fat content than after oviposition (Lipsitz and McFarlane, 1971; Nestel et al., 2005). Insects collected from the wild seem to have a lower fat content than insects which are commercially produced (Finke, 2002, 2013; Oonincx and Dierenfeld, 2012; Yang et al., 2006). This might be a result of decreased movement in captivity, easy access to high energy diets or a combination of the two.

Fatty acids are the building blocks of fat. Two or three fatty acids are coupled to glycerol to form diglycerides and triglycerides respectively. These fatty acids are stored in the insect fat body, making up over 90% of the total fat body lipid (Beenakkers et al., 1985; Bender, 2002; Downer and Matthews, 1976). Based on the degree of saturation, fatty acids can be subdivided into saturated fatty acids (those with no double bonds) and unsaturated fatty acids (those with one (mono-unsaturated fatty acids) or more (poly-unsaturated fatty acids) double bonds). Poly-unsaturated fatty acids can be further subdivided into omega 3, 6, or 9 unsaturated fatty acids based on the relative position of the first double bond. Both the absolute occurrence of unsaturated fatty acids (Haglund et al., 1998) and the relative occurrence of specific unsaturated fatty acids (Schmitz and Ecker, 2008) are associated with health in humans, and these proportions might also play a role in the health of some species of insectivores.

For most insect species, more than half of their fatty acids are unsaturated, a notable exception being the Hemiptera (Thompson, 1973). The main saturated fatty acids found in insects are C16:0 (palmitic acid) and C 18:0 (stearic acid). As is observed with most other land animals, C16:0 is normally present in larger quantities than C18:0 (Fast, 1970; Majumder and Sengupta, 1979; Thompson, 1973; Yang et al., 2006). The most prevalent unsaturated fatty acids found in insects are C16:1 (palmitoleic acid), C18:1 (oleic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid) (Beenakkers et al., 1985; Bukkens, 1997; Cookman et al., 1984; Ekpo et al., 2009; Fast, 1970; Majumder and Sengupta, 1979; Yang et al., 2006).

The fatty acid composition of insects is affected by four main variables: (1) species, (2) developmental phase, (3) diet, and (4) environmental factors such as temperature, light, and humidity.

Phylogeny is not the main determining factor for fatty acid composition, although some general distinctions can be made (Fast, 1970; Fontaneto et al., 2011). For instance, aphids and other Hemiptera tend to contain large amounts of the short-chained fatty acids C12:0 (lauric acid), and C14:0 (myristic acid) (Fast, 1970; Thompson, 1973). Lepidopterans tend to have a higher C18:3 content than other insect species (Fast, 1970; Fontaneto et al., 2011; Majumder and Sengupta, 1979), while Dictyoptera, such as cockroaches, contain little or no C18:3 (Finke, 2013; Thompson, 1973). In most species of Diptera C16:0, C16:1, and 18:1 predominate although C18:2 is also present in high levels in gall midges (Cecidomyiidae) (Fast, 1970; Thompson, 1973). Black soldier fly larvae contain high levels (up to 79% of the total fatty acids) of C12:0 although the exact amounts are determined by the insect's diet and life stage (Danieli et al., 2019; Ewald et al., 2020; Giannetto et al., 2020a; Hoc et al., 2020; Liu et al., 2017; Meneguz et al., 2018; Oonincx et al., 2019; Spranghers et al., 2017; St-Hilaire et al., 2007). Of note is that lauric acid has antimicrobial properties and the inclusion of black soldier fly fat in place of soybean oil into diets for young turkeys reduces the proliferation of potentially pathogenic bacteria (Sypniewski et al., 2020).

The fatty acid patterns of aquatic and terrestrial insects differ presumably because of their diet. Aquatic insects have relatively high levels of long-chain omega-3 fatty acids, in particular, 20:5 (eicosapentaenoic acid) and 22:6 (docosahexaenoic acid), which are rarely found in terrestrial insects (Fontaneto et al., 2011; Ghioni et al., 1996; Sushchik et al., 2003, 2013).

Insects contain higher amounts of C18:3 than most mammals. Like vertebrates, de novo synthesis of saturated fatty acids and poly-unsaturated fatty acids by elongation and desaturation occurs in insects (Beenakkers et al., 1985, Tietz and Stern, 1969). Certain poly-unsaturated fatty acids such as C18:2 and C18:3 are required in the diet because most animal species, including most insects, are unable to synthesize them (Bender, 2002; Cookman et al., 1984; Fast, 1970; Thompson, 1973). A few noticeable exceptions have been identified, for example, the American cockroach (*Periplaneta americana* L.) and the house cricket (Beenakkers et al., 1985; Blomquist et al., 1991). Several studies have shown that C18:2 can be synthesized de novo in these and other insect species, distributed over four different orders (Blomquist et al., 1991; Borgeson et al., 1991; Borgeson and Blomquist, 1993; de Renobales et al., 1987).

18.2.3 Carbohydrates

Few publications have focused on the carbohydrate content of insects. In general, carbohydrates are present in relatively small amounts in insects (Finke, 2002, 2013). In the two-spotted field cricket (*Gryllus bimaculatus* De Geer), polysaccharide and carbohydrate content are approximately 0.3% of the fresh weight (Hoffmann, 1973) and free carbohydrate content of the fat body in females of this species is less than 0.5% dry matter (Lorenz and Anand, 2004). In yellow mealworm larvae, the carbohydrate content can vary between 1% and 7% dry matter, depending on the diet provided (Ramos-Elorduy et al., 2002) although most of these differences are likely the result of food remaining in the gastrointestinal tract.

18.2.4 Fiber and chitin

Insects contain significant amounts of fiber as measured by crude fiber (CF), acid detergent fiber (ADF) and neutral detergent fiber (NDF) (Barker et al., 1998; Finke, 1984, 2002, 2007, 2013; Lease and Wolf, 2010; Oonincx and Dierenfeld, 2012; Pennino et al., 1991; Punzo, 2003). While insects contain significant amounts, the components that make up these fibers are unknown. It has been suggested that the fiber in insects represents chitin, since chitin (linear polymer of β-(1→4) N-acetyl-D-glucosamine units) is structurally similar to cellulose (linear polymer of β-(1→4)-D-glucopyranose units) found in plant material. In reality the fiber in whole insects likely represents a variety of different compounds including chitin, sclerotized proteins and other substances that are bound to chitin (Finke, 2007). Chitinase activity has been reported in certain species of frogs, lizards, tortoises, fish, birds and mammals suggesting that certain insectivores might be able to digest chitin (Donoghue, 2006; Fujimoto et al., 2002; German et al., 2010; Jackson et al., 1992; Jeuniaux and Cornelius, 1978; Lindsay, 1984; Tabata et al., 2018; Whitaker et al., 2004).

In insects, chitin exists in a matrix with proteins, lipids, and other compounds which together comprise the cuticle (Kramer et al., 1995). This matrix makes it difficult to analyze pure chitin in insects and to provide good quantitative estimates. However, since chitin is present only in the insect's exocuticle its' concentration is likely low. Methods used to estimate insect chitin include a modified CF analysis (Liu et al., 2012; Woods et al., 2020), ADF (Barker et al., 1998), ADF corrected for residual amino acids (Finke, 2007, 2013; Giannetto et al., 2020a), digestion by sodium

hydroxide (Lease and Wolf, 2010), glucosamine determination after the breakdown of chitin (Cauchie, 2002; Henriques et al., 2020) and calcofluor staining (Henriques et al., 2020). In most studied insects, protein, not chitin is the predominant compound in the cuticle (Kramer et al., 1995). The amino acid patterns from whole insects differ from their ADF fractions and these patterns in ADF fractions also differ between species (Finke, 2007). These differences likely reflect specificity in insect cuticular proteins, which contribute to their unique properties.

While "harder bodied" insects like adult beetles contain higher levels of ADF than softer bodied insects like yellow mealworm larvae, silkworm larvae or cricket nymphs, those were due to higher levels of amino acids in the ADF fraction (Finke, 2007). This suggests that insects with "harder" cuticles do not necessarily contain more chitin than softer bodied insects, but rather contain higher levels of cross-linking proteins that are essential for sclerotization.

Some plant-based fibers can function as prebiotics inhibiting the growth of pathogenic bacteria and promoting the growth of beneficial ones. Little is known regarding the effects of fiber from insects on insectivores, although some research suggests pigs, poultry and fish fed diets containing dried insect meals have improved gut microbiota or immune function (Bruni et al., 2018; Ringø et al., 2012; Schiavone et al., 2017; Spranghers, et al., 2018). The exact components in these meals responsible for these effects are however unknown.

18.2.5 Minerals

Minerals can be broadly classified as macro-minerals and micro- or trace minerals based on the amounts needed to meet requirements. The essential macrominerals include calcium, phosphorus, magnesium, sodium, potassium and chloride. The essential microminerals include iron, zinc, copper, manganese, iodine, and selenium. The macrominerals calcium, phosphorus and magnesium play a primary role in helping maintain the skeletal structure in vertebrates while sodium, potassium and chloride function as electrolytes and help maintain acid−base balance. The trace minerals play wide-ranging roles ranging from oxygen transport to functioning as cofactors in a variety of enzyme systems.

Most species of insects contain little calcium because insects, as invertebrates, do not have a mineralized skeleton. Calcium levels are typically less than 0.3% dry matter (Barker et al., 1998; Finke, 2002, 2013; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003; Reichle et al., 1969; Studier and Sevick, 1992). The higher levels of calcium occasionally reported for feeder crickets likely reflect calcium in the gut contents (Barker et al., 1998; Finke, 2003; Hatt et al., 2003; Punzo, 2003). The exoskeleton of most insects is primarily composed of protein and chitin, although some insects have a mineralized exoskeleton in which calcium and other minerals are incorporated into the cuticle (Dashefsky et al., 1976). Examples include larvae of the face fly, *Musca autumnalis* De Geer, and the black soldier fly (Boykin et al., 2020; Dierenfeld and King, 2008; Finke, 2013). Calcium levels reported for commercially available black soldier fly larvae ranged from 2.14% to 3.14% with calcium:phosphorus ratios of 1.85 to 2.6:1 (Boykin et al., 2020; Dierenfeld and King, 2008; Finke, 2013). However, black soldier fly larvae fed various waste products were even more variable with calcium levels ranging from 0.84% to 8.29% dry matter and with calcium:phosphorus ratios of 1.1:1 to 4.7:1 (Liland et al., 2017; Tschirner and Simon, 2015). Therefore, reliable data on the calcium content of specific sources and batches is needed before black soldier fly larvae can be considered a reliable source of calcium for insectivores. Moreover, besides calcium content, its' ratio with phosphorus is of relevance to facilitate adequate bone mineralization in insectivores. Other invertebrates such as millipedes and isopods also have a mineralized exoskeleton and likely serve as a source of calcium for wild insectivores (Gist and Crossley, 1975; Graveland and van Gijzen, 1994; Oonincx and Dierenfeld, 2012; Reichle et al., 1969). Another way of supplying calcium might be offering calcium grit, powder, or other calcium rich materials separately to insectivores to allow self-selection (Classen and Scott. 1982; Rich and Talent, 2008; Tordoff, 2001). Wild birds seek out calcium-rich invertebrates when calcium requirements are high, such as during egg-laying and nestling growth (Graveland and van Gijzen, 1994; Bureš and Weidinger, 2003).

The phosphorus content of feeder insects is much higher than calcium levels in most insect species, except for black soldier fly larvae (Barker et al., 1998; Finke, 2002, 2013; Hatt et al., 2003; Jones et al., 1972; Martin et al., 1976; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). Most insects likely contain adequate levels of phosphorus to meet the requirements of insectivores. To what extent this phosphorus is available is unclear. Using tibia ash as the criteria for phosphorus availability relative to dicalcium phosphate, 92% of the phosphorus in dried face fly pupa (*M. autumnalis* DeGeer) was available when fed to poultry (Dashefsky et al., 1976). In contrast, only 24% of the phosphorus in a diet containing dried black soldier fly larva was absorbed when fed to newly weaned five-week-old pigs compared to 51% for a corn-soy diet (Newton et al., 1977). Of note is that the diet containing dried back soldier fly larvae also contained other ingredients (corn and dicalcium phosphate) which provided significant amounts of phosphorus making it impossible to definitively determine the phosphorus availability from the dried back soldier fly larvae.

Most species of feeder insects contain levels of magnesium ranging from 0.08% to 0.40% dry matter (Barker et al., 1998; Finke, 2002, 2013; Jones et al., 1972; Martin et al., 1976; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). These levels would likely meet the dietary requirements of most insectivore species. Like calcium, the magnesium content of black soldier fly larvae was 3−10 times higher than that of other feeder insects (Boykin et al., 2020; Dierenfeld and King, 2008; Finke, 2013). It seems likely that both calcium and magnesium form a complex with chitin in the larval cuticle of this species.

There are few reports on the sodium and potassium content of captive-bred insects (Finke, 2002, 2013, 2015a; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012), but these data are comparable to the values obtained for wild-caught insects (Finke, 2015b; Gist and Crossley, 1975; Levy and Cromroy, 1973; Oyarzun et al., 1996; Reichle et al., 1969; Studier et al., 1991; Studier and Sevick, 1992). Levels of potassium generally range from 0.6%−2.0% dry matter, while sodium levels are lower ranging from 0.1% to 0.6% dry matter. There are limited data concerning the chloride content of feeder insects with values ranging from 0.16% to 0.97% dry matter (Finke, 2002, 2013, 2015a, 2015b). These data suggest that most insects likely contain adequate amounts of these three minerals to meet the needs of most insectivore species (Finke, 2002, 2013; Oonincx and Dierenfeld, 2012).

Most insects appear to contain relatively high levels of the trace minerals iron, zinc, copper and manganese. Additionally, iron, copper, zinc, and manganese availability from grasshoppers (*Sphenarium purpurascens* Charpentier), two-spotted field crickets, yellow mealworm larvae, and lesser mealworm larvae as measured by an in vitro assay was higher than that observed for beef suggesting these minerals are likely readily available to insectivores (Latunde-Dada et al., 2016).

While the high-fat larval stage of some species of feeder insects like greater wax moth larvae, yellow mealworm larvae and butterworm larvae might be marginally low in iron, relative to energy content, most insects likely supply adequate amounts for the typical insectivore (Barker et al., 1998; Finke, 2002, 2013, 2015a; Hatt et al., 2003; Mwangi et al., 2018; Montowska et al., 2020; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). Both adult houseflies and fruit flies (*Drosophila melanogaster* Meigen) contain relatively high levels of iron (125−454 mg iron/kg dry matter) (Barker et al., 1998; Finke, 2013; Oonincx and Dierenfeld, 2012). House crickets can contain up to 200 mg iron/kg dry matter although other reports documented iron content to be between 60 and 80 mg/kg dry matter (Barker et al., 1998; Bernard and Allen, 1997; Finke, 2002, 2015a). These variations might be due to food remaining in the gastro-intestinal tract when the insects were analyzed, although potential contamination during processing should also be considered. Wild-caught insects also appear to contain significant amounts of iron (Finke, 2015b; Levy and Cromroy, 1973; Punzo, 2003; Studier et al., 1991; Studier and Sevick, 1992).

Insects are generally a good source of zinc with values for commercially raised feeder insects ranging from 61.6 to 340.5 mg/kg dry matter (Barker et al., 1998; Finke, 2002, 2013, 2015a; Latunde-Dada et al., 2016; Montowska et al., 2020; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). These values are similar to those obtained for wild-caught species (Finke, 2015b; Punzo, 2003).

Copper in commercially raised feeder insects ranged from 3.1 to 51.2 mg/kg dry matter (Barker et al., 1998; Finke, 2002, 2013, 2015a; Latunde-Dada et al., 2016; Montowska et al., 2020; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). The lowest value seen in captive-raised insects was 3.1 mg copper/kg dry matter for greater wax moth larvae reported by Barker (Barker et al., 1998) while Finke (2002) found a much higher level for this species (9.2 mg copper/kg dry matter). All other species analyzed had values greater than 7 mg copper/kg dry matter suggesting insects are typically good sources of copper. Wild-caught insects also appear to contain significant amounts of copper (Finke, 2015b; Levy and Cromroy, 1973; Punzo, 2003).

Levels of manganese in commercially raised feeder insects range from 1.5 to 364 mg/kg dry matter (Barker et al., 1998; Bernard and Allen, 1997; Dierenfeld and King, 2008; Finke, 2002, 2013, 2015a; Latunde-Dada et al., 2016; Montowska et al., 2020; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012; Punzo, 2003). Like calcium and magnesium, the highest levels of manganese observed were seen in black soldier fly larvae (Boykin et al., 2020; Dierenfeld and King, 2008; Finke, 2013) although the reasons for elevated levels of manganese in this species are unclear. Several species of stored product insects contain elevated levels of zinc and manganese in their mandibles, presumably to enable them to penetrate whole seeds (Morgan et al., 2003). Wild-caught insects also appear to contain significant amounts of manganese (Finke, 2015b; Punzo, 2003).

There are very little data regarding the iodine content of insects. Of the twelve species of commercially raised feeder insects analyzed only six had detectable iodine with levels ranging from 0.45 to 1.22 mg/kg dry matter (Finke, 2002, 2013, 2015a). Black soldier fly larvae fed graded levels of iodine-rich (700 mg/kg dry matter) seaweed contained increasing levels of iodine (0−260 mg /kg dry matter) (Liland et al., 2017). Since these authors harvested fully fed larvae at least some of these increases are likely a result of the food remaining in the gastrointestinal tract. Bee

brood (pupae and larvae) and wild-caught white-lined sphinx moths (*Hyles lineata* Fabricius) did not contain detectable levels of iodine while wild-caught pallid winged grasshoppers (*Trimerotropis pallidipennis* Burmeister) and rhinoceros beetles (*Oxygrylius ruginasus* LeConte) contained 0.89 and 0.46 mg I/kg dry matter respectively (Finke, 2005, 2015b).

As is the case for iodine, only limited data on the selenium content of commercially raised feeder insects are available. While butterworm larvae did not contain any selenium the other eleven species of commercially raised feeder insects contained selenium at levels ranging from 0.27 to 0.97 mg/kg dry matter (Finke, 2002, 2013, 2015a). Three species of wild caught insects contained similar levels (0.55−0.75 mg Se/kg dry matter) as those reported for commercially raised feeder insects (Finke, 2015b). In contrast, Indonesian sago grub (larvae of the beetle *Rhynchophorus bilineatus* Montrouzier) contained no detectable selenium (Köhler et al., 2020). As selenium can bioaccumulate in insects, it seems likely that concentrations are strongly influenced by dietary levels (Arnold et al., 2014).

Since the contents of the gastrointestinal tract can represent a significant percentage of the total weight of the insect (Finke, 2003), it can have a significant effect on the mineral content of the insect if analyzed when fully fed. Additionally, studies on wild-caught insects show both seasonal variations as well as variation between different populations of the same species living in the same general area (Finke, 1984; Studier et al., 1991; Studier and Sevick, 1992).

18.2.6 Vitamins and carotenoids

18.2.6.1 Vitamin A

Vitamin A plays a role in a wide variety of physiological processes including vision, cell differentiation, immune response, reproduction and growth. There are limited data regarding the vitamin A content of wild-caught insects and most captive-bred insects contain relatively low levels of vitamin A/retinol (typically less than 300 μg retinol/kg dry matter) (Barker et al., 1998; Bawa et al., 2020b; Finke, 2002, 2013; Hatt et al., 2003; Oonincx and van der Poel, 2011; Parker et al., 2020; Pennino et al., 1991; Punzo, 2003). Migratory locusts (*Locusta migratoria* L.) fed a grass diet supplemented with wheat bran and fresh carrots contained significantly more retinol than those fed only a grass diet (Oonincx and van der Poel, 2011). However, the retinol levels for all locusts were well below the requirements of the rat.

In fruit flies only the eyes contained measurable quantities of retinoids and the amount detected was a function of the carotenoid content of the larval diet (Giovannucci, Stephenson, 1987; Goldsmith and Warner, 1964; Seki et al., 1998; Von Lintig, 2012). The low values reported in the literature for the vitamin A content of captive-bred insects may be a result of several factors. First, most analytical methods used for vitamin A analysis are specific to retinol and may not detect other retinoids (retinal and 3-hydroxyretinal) found in insect eyes (Goldsmith and Warner, 1964; Seki et al., 1998; Smith and Goldsmith, 1990). It is unclear if 3-hydroxyretinal can serve as a source of vitamin A since it is unknown if 3-hydroxyretinal can be converted into retinal by insectivores. Second, retinoid levels in fruit fly eyes are a function of dietary carotenoid content and diets fed to commercially raised insects may not contain sufficient levels or the correct types of carotenoids to optimize retinal and 3-hydroxyretinal content of insects. Third, many insect species raised for food are fed to insectivores as larvae, which do not possess compound eyes where retinoid synthesis from carotenoids takes place (Von Lintig, 2012).

In addition to retinol, certain carotenoids can be converted into vitamin A in many animal species (Bender, 2002; Levi et al., 2012; McComb, 2010; Olson, 1989). Although it is unclear if all insectivores have the ability to convert beta-carotene and other pro-vitamin A carotenoids to retinol, both leopard geckos (*Eublepharis macularius* Blyth) (Cojean et al., 2018) and Mississippi gopher frogs (*Rana sevosa* Goin and Netting) (Ploog et al., 2015) seem to be able to convert beta-carotene to retinol. Most captive-bred insects contain little if any carotenoids although in a few instances moderately high levels have been reported (Finke, 2002, 2013, 2015a; Jones et al., 1972; Oonincx and van der Poel, 2011; Oonincx and Dierenfeld, 2012). In contrast, high levels of carotenoids are found in wild-caught insects (Arnold et al., 2010; Cerda et al., 2001; Eeva et al., 2010; Helmer et al., 2015; Isaksson and Andersson, 2007; Maoka et al., 2020, 2021; Newbrey et al., 2013; Ssepuuya et al., 2017). The reason for this discrepancy is likely a function of dietary carotenoid intake, as was shown in both fruit flies (Giovannucci and Stephenson, 1999) and silkworm larvae (Chieco et al., 2019). Wild insectivores likely use a combination of retinoids (retinal and possibly 3-hydroxyretinal) and pro-vitamin A carotenoids to meet their vitamin A requirements. A better understanding of the retinoid content of insects and the potential conversion of various retinoids and carotenoids to vitamin A by insectivores is important since vitamin A deficiency has been reported in captive insectivores (Ferguson et al., 1996; Hoby et al., 2010; Miller et al., 2001; Pessier et al., 2005; Wiggans et al., 2018).

18.2.6.2 Vitamin D

Vitamin D_3 is a conditionally essential nutrient for the majority of insectivores as most animal species can synthesize it de novo provided, they are exposed to adequate amounts of UVb radiation (Bos, et al., 2018; Diehl et al., 2018; Oonincx et al., 2018, 2020; Schutkowski et al., 2013; Watson et al., 2014). As most commercially produced insects lack access to UVb, their vitamin D content depends on dietary intake. None of the five commercially produced insect species reached the threshold for detection of 250 IU/kg fresh matter (~595–1445 IU/kg dry matter) (Finke, 2002). Using a more sensitive technique, black soldier fly larvae, butter worm larvae and Turkestan roaches were shown to contain 388–633 IU vitamin D_3/kg dry matter, while adult houseflies were still below the limit of detection (Finke, 2013). A further study reports a low vitamin D_3 level in commercially produced yellow mealworms (150 IU/kg dry matter), but a higher concentration in house crickets (934 IU/kg dry matter) (Oonincx et al., 2010). These highly variable concentrations were attained via dietary accumulation. In wild-caught specimens with the opportunity to expose themselves to UVb, vitamin D_3 levels are highly variable. For instance, wild-caught ant eggs and adult rhinoceros beetles can contain high levels (1288–2806 IU/kg dry matter), whereas these seem lower in pallid-winged grasshoppers (284 IU/kg dry matter) and far lower (<187 IU vitamin D_3) for white-lined sphinx moths (Finke, 2015b; Melo-Ruiz et al., 2013). While further studies are needed to provide insight into the mechanisms behind these variable concentrations, access to UVb plays a role. Providing UVb access to four insect species showed increases in vitamin D_3 concentrations in house crickets (121–361 IU/kg dry matter), migratory locusts (0–557 IU/kg dry matter) and yellow mealworms (163–6973 IU/kg dry matter) as a result of UVb exposure (Oonincx et al., 2018). Conversely, this effect was absent in black soldier fly larvae. Hence, it seems that UVb exposure can make certain feeder insects a significant dietary source of vitamin D_3, although not for others. However, it seems plausible that providing UVb to the insectivores themselves may be a suitable way to ensure an adequate vitamin D status.

18.2.6.3 Vitamin E

Vitamin E serves as an antioxidant and therefore helps maintain the functionality of a variety of lipid-soluble compounds in the body. Insects contain varying amounts of vitamin E. House crickets, yellow mealworms and black soldier fly larvae can contain widely varying levels of vitamin E. Values for house crickets range from 5–131 mg vitamin E/kg dry matter (Barker et al., 1998; Finke, 2002, 2015a; Hatt et al., 2003; Pennino et al., 1991) yellow mealworm larvae range from less than 15 mg/kg to 78 mg vitamin E/kg dry matter (Barker et al., 1998; Finke, 2002, 2015a; Pennino et al., 1991) and black soldier fly larvae range from 6.7 to 104 mg vitamin E/kg dry matter (Finke, 2013; Liland et al., 2017; Liu et al., 2017; Shumo et al., 2019). The large variations appear to be a function of the insect's diet, representing both the vitamin E incorporated into the body tissue as well as the vitamin from the diet remaining in the insect's gastrointestinal tract. The vitamin E content of other commercially raised feeder insects is relatively low (typically less than 15 mg/kg dry matter) (Barker et al., 1998; Finke, 2002, 2013; Jones et al., 1972; Oonincx and Dierenfeld, 2012; Punzo, 2003). Several species, including butterworm larvae and silkworm larvae have somewhat higher levels (33–35 mg vitamin E/kg dry matter) while much higher levels (110–120 mg vitamin E/kg dry matter) are reported for fruit flies, house flies and false katydids (*Microcentrum rhombifolium* Saussure) (Finke, 2002, 2013; Oonincx and Dierenfeld, 2012). Wild-caught insects appear to contain vitamin E at levels on the higher end of the range or exceeding those seen in captive-bred insects (Arnold et al., 2010; Cerda et al., 2001; Finke, 2015b; Fogang et al., 2017; Köhler et al., 2020; Koštál et al., 2013; Pennino et al., 1991; Punzo, 2003; Teffo et al., 2007).

18.2.6.4 B-vitamins

While there is limited comprehensive information regarding the B-vitamin content of most insects, there are reports on the B-vitamin content of the most commonly produced feeder insects (Bawa et al., 2020a; Finke, 2002, 2013, 2015a; Jones et al., 1972). As data on B-vitamin content of unprocessed, wild-caught insects is lacking, it is unclear whether captive-bred and wild-caught insects have similar levels. Furthermore, differences in analytical methods (microbiological vs chemical techniques) and in methods for preparing samples further complicate direct comparisons. Some B-vitamins are relatively unstable when exposed to heat, light or oxygen and can be lost during sample preparation. Therefore, data from dried insects processed for human consumption may underestimate the concentrations in live whole insects. For the same reason, commercially available dried insects may contain lower levels of some B-vitamins due to processing, drying and storage. The data that is available is summarized below.

Thiamine (vitamin B_1) is needed as a cofactor for several important enzymes associated with energy metabolism (Thurnham et al., 2000). A number of species of feeder insects contain fairly low levels of thiamine including house crickets (<0.2–0.7 mg thiamine/kg dry matter), adult yellow mealworms (1.7 mg thiamine/kg dry matter), superworms

(1.4−4.6 mg thiamine/kg dry matter), butterworms (1.8 mg thiamine/kg dry matter), and Turkestan roaches (2.9 mg thiamine/kg dry matter) (Bawa et al., 2020b; Finke, 2002, 2013, 2015a). Other species of feeder insects including black soldier fly larvae (19.8 mg/kg dry matter), adult house flies (44.8 mg thiamine/kg dry matter), silkworms (19.1 mg thiamine/kg dry matter), yellow mealworm larvae (3.5−6.3 mg thiamine/kg dry matter), and waxworms (3.3−5.5 mg thiamine/kg dry matter) contain higher levels (Finke, 2002, 2013, 2015a). A wide range of thiamine levels (0.7−14.3 mg thiamine/kg dry matter) has also been reported for wild-caught rhinoceros beetles, pallid-winged grasshoppers and adult white-lined sphinx moths (Finke, 2015b). Similarly, large variations (0.3−32.4 mg thiamine/kg dry matter) are reported for oven-dried wild-caught Nigerian insects comprising five orders (Banjo et al., 2006). Based on a microbiological method, high thiamine levels (30.2 and 36.7 mg thiamine/kg dry matter) are reported for African palm weevil larva [*Rhynchophorus phoenicis* (F.)] and larvae of the cavorting emperor moth (*Usta terpsichore* (Maassen and Weymer)), while the values for termites [*Macrotermes subhyalinus* (Rambur)] were far lower (1.3 mg thiamine/kg dry matter) (Santos Oliveira et al., 1976). Similarly low levels of thiamine (1.5−2.7 mg thiamine/kg dry product) are reported based on a microbiological method for dried and smoked Attacidae caterpillars from Zaire (Kodondi et al., 1987). As thiamine is relatively unstable and dried insects were processed using a variety of traditional methods (drying, smoking, and frying), unprocessed insects might have higher concentrations (Banjo et al., 2006; Kodondi et al., 1987; Santos Oliveira et al., 1976; Teffo et al., 2007).

Riboflavin (vitamin B_2) is a co-enzyme in the metabolism of a variety of nutrients (Thurnham et al., 2000). Most species of commercially raised feeder insects contain relatively high levels of riboflavin ranging from 17.6 to 306.3 mg/kg dry matter (Bawa et al., 2020b; Finke, 2002, 2013, 2015a; Jones et al., 1972). Similar levels (32.4−39.2 mg riboflavin/kg dry matter) were found for honeybee brood (larvae and pupae) (Banjo et al., 2006; Finke, 2005) and wild-caught rhinoceros beetles, pallid-winged grasshoppers and adult white-lined sphinx moths (66.5−82.7 mg riboflavin/kg dry matter) (Finke, 2015b). Dried and smoked Attacidae caterpillars also contain high levels of riboflavin (32−51 mg/kg dry product) (Kodondi et al., 1987). These values are higher than those for termites, palm weevil larvae and a saturnid caterpillar processed for human consumption (9.0−22.4 mg riboflavin/kg dry matter) (Santos Oliveira et al., 1976; Teffo et al., 2007). Banjo reports highly variable levels of riboflavin in 14 species of insects from Nigeria ranging from 0.9 to 32.4 mg/kg dry matter (Banjo et al., 2006). After collection, these samples were kept dry (adults) or stored in 70% alcohol (larvae). Since riboflavin is degraded by light it is unclear if these represent real differences or are a result of losses during storage prior to analysis.

Niacin (vitamin B_3) plays a role in metabolism and tissue respiration (Thurnham et al., 2000). It appears abundant in commercially raised feeder insects, with levels ranging from 77 to 359 mg/kg dry matter (Finke, 2002, 2013, 2015a). Bawa et al. (2020b) obtained much lower values (3.6−30.1 mg niacin/kg dry matter) for house crickets compared to previous studies (107−143 mg niacin/kg dry matter) (Finke, 2002, 2015a). The reason for these discrepancies is unknown but may be related to analytical methods. Wild-caught rhinoceros beetles, pallid-winged grasshoppers and adult white-lined sphinx moths also contained high levels of niacin (96.4−191.4 mg niacin/kg dry matter) (Finke, 2015b). A variety of dried insects consumed in Africa, including termites, palm weevil larvae and several species of caterpillars, have been analyzed for niacin. Even though these were processed with a variety of methods (drying, smoking and frying) all had high levels of niacin, ranging from 52 to 110 mg/kg dry matter (Kodondi et al., 1987). Conversely, larvae of the cavorting emperor moth contain only 3 mg niacin/kg dry matter (Santos Oliveira et al., 1976). As niacin is a relatively stable B-vitamin these values are likely representative of live insects.

Pantothenic acid (vitamin B_5) functions as a component of coenzyme A and as such plays a role in the citric acid cycle, fatty acid synthesis and oxidation reactions. It is widely distributed in most foodstuffs and commercially raised insects appear to be no exception, with levels ranging from 18.9 to 179.8 mg/kg dry matter (Finke, 2002, 2013, 2015a). Bee brood, wild-caught rhinoceros beetles, pallid-winged grasshoppers, and adult white-lined sphinx moths also appear to be good sources of pantothenic acid (41.8 to 56.5 mg pantothenic acid/kg dry matter) (Finke, 2005, 2015b). There are little additional data available on the pantothenic acid content of wild-caught insects although using a microbiological assay dried smoked Attacidae caterpillars were found to contain very low levels of pantothenic acid (0.073−0.102 mg/kg dry product) (Kodondi et al., 1987).

Pyridoxine (vitamin B_6) plays an important role in several metabolic reactions including amino acid metabolism. Commercially raised insects appear to be good sources of pyridoxine with values ranging from 1.3 to 4.8 mg pyridoxine/kg dry matter for waxworm larvae to 22.3 mg /kg dry matter for yellow mealworm beetles (Bawa et al., 2020b; Finke, 2002, 2013, 2015a; Jones et al., 1972). Most insect species, including three species of wild-caught insects, contain between 5 and 10 mg pyridoxine/kg dry matter. Very low levels of pyridoxine (0.37 to 0.63 mg/kg) were detected in three species of dried smoked Attacidae caterpillars using a microbiological technique (Kodondi et al., 1987). These low values might be due to the smoking and drying processes used to preserve these insect products as pyridoxine is susceptible to degradation by heat.

Biotin (vitamin B_8) carries carboxyl groups in ATP reactions. The biotin content of both commercially raised and wild-caught insects is highly variable ranging from 0.17 to 2.69 mg/kg dry matter, however, the values for most species ranged from 0.7 to 1.4 mg/kg dry matter (Finke, 2002, 2013, 2015a). Dried smoked meal from Attacidae caterpillars has been reported to contain 0.23−0.45 mg/kg (Kodondi et al., 1987). It is unclear if these low values reflect biological differences, biotin loss during processing (drying and smoking), or analytical techniques used.

Folic acid (vitamin B_9) plays an essential role in DNA synthesis and one carbon metabolism (Thurnham et al., 2000). Using a chemical method, commercially raised insects were shown to contain high levels of folic acid ranging from a low of 1.38 mg/kg for waxworm larvae to 7.22 mg/kg for house flies (Finke, 2002, 2013, 2015a). Lower values were observed for three species of wild-caught insects (0.17 to 2.4 mg folic acid/kg dry matter). In contrast, very low folic acid values (0.20−0.63 mg folic acid/kg dry matter) were found for smoked and dried product from three species of Attacidae caterpillars using a microbiological method (Kodondi et al., 1987). Since folic acid is susceptible to degradation both by light and oxidizing agents the low values may be due to the smoking and drying process used to preserve the insect product.

Cobalamin (vitamin B_{12}) is found exclusively in animal products and plays a key role in reactions involving methyl donors and concentrations in insects vary greatly. At the lower end, are commercially raised silkworm larvae, waxworm larvae and wild-caught adult sphinx moths, with levels below 3−4 μg/kg dry matter (limit of detection) (Finke, 2002, 2015b). These are followed by low levels in yellow mealworm larvae and beetles, as well as superworms (1.3−15 μg/kg/dry matter; Finke, 2002, 2015a; Lenaerts et al., 2018; Schmidt et al., 2019). Levels in Jamaican field crickets are somewhat higher (28.8 μg/kg dry matter; Schmidt et al., 2019), but not as high as most house crickets (174−702 μg/kg dry matter; Finke, 2002, 2015a). Although data is limited, it seems that black soldier fly larvae (144 μg/kg dry matter; Finke, 2013) and Turkestan cockroaches (132−767 μg/kg dry matter) are both good sources of vitamin B_{12} (Finke, 2013; Schmidt et al., 2019). Similarly, high vitamin B_{12} levels (140−250 μg/kg dry matter) are reported for dried and smoked products from three species of Attacidae caterpillars (Kodondi et al., 1987).

18.2.6.5 Vitamin C

Vitamin C is needed to form connective tissue and functions as an antioxidant. Insects contain some vitamin C and while some are able to synthesize it de novo, others depend on nutritional sources (Kramer and Seib, 1982). Honeybees contain relatively high amounts of vitamin C (103 to 164 mg/kg dry matter) (Banjo et al., 2006). Whereas both adult house crickets and adult mealworms contain similar levels (97−149 mg/kg dry matter), most other species contain less than 50 mg/kg dry matter (Finke, 2002, 2007, 2013; Banjo et al., 2006).

18.2.7 Other nutrients

In addition to the aforementioned nutrients, insects contain a variety of other compounds that may be important to help maintain the health of insectivores. These include choline, taurine, and various sterols including cholesterol (Finke, 2015a, 2015b; Cerda et al., 2001; Ramsay and Houston, 2003).

18.2.7.1 Choline

Choline is a component of both lecithin and the neurotransmitter acetylcholine and also plays an important role in one-carbon metabolism (Pesti et al., 1979). As such it can spare the need for dietary methionine an important consideration given that methionine is typically the first-limiting amino acid in most insect species as was noted earlier. While most reported choline analysis is from commercially raised feeder insects, the data shows insects contain high levels of choline (1570−7258 mg/kg dry matter) (Finke, 2002, 2013, 2015a, 2015b; Fogang et al., 2017; Noland and Baumann, 1949).

18.2.7.2 Taurine

Taurine, an amino sulfonic acid, is involved in cell volume regulation, provides a substrate for the formation of bile salts, and plays a role in the regulation of intracellular calcium. It is a required nutrient for some species, such as cats and foxes (Hayes et al., 1975; Moise et al., 1991), but most species can synthesize taurine from methionine provided sufficient precursors are available. The only insectivore studied, the giant anteater (*Myrmecophaga tridactyla* L.) has been shown to synthesize taurine from dietary methionine (Nofs et al., 2018). House crickets, grasshoppers, adult fruit flies, house flies, and moths are rich sources of taurine containing (2800−7300 mg/kg). In contrast adult beetles and most species of insect larvae contain little if any taurine (Bodnaryk, 1981; Finke, 2002, 2013, 2015a, 2015b; Giannetto et al., 2020b; Massie et al., 1989; Ramsay and Houston, 2003). The highest levels of taurine in insects are found in the

flight muscles which is likely why most adult holometabolous insects contain high levels of taurine (Whitton et al., 1987). It has been proposed that adult passerines have a preference for spiders (7200−21,350 mg taurine/kg dry matter), driven by a need for enhanced levels of methionine, cystine, or taurine by their growing chicks (Ramsay and Houston, 2003). Supplementing the diet of wild blue tit (*Cyanistes caeruleus* L.) nestlings from 2 to 12 days of age with taurine resulted in juveniles that took significantly greater risks when investigating novel objects and were more successful at a spatial learning task than controls (Arnold et al., 2007). The significance of dietary taurine for other species of insectivores is unknown.

18.2.7.3 Sterols

Sterols play a critical role as structural components in the phospholipid bilayer of cell membranes. The dominant sterols in most insects are β-sitosterol, 7-dehydrocholesterol and cholesterol, although insects contain a variety of other sterols including campesterol and stigmasterol (Cerda et al., 2001; Connor et al., 2006; Jing and Behmer, 2020; Koštál et al., 2013; Liland et al., 2017; Mlček et al., 2019; Sabolová et al., 2016; Svoboda et al., 1995). Both the total sterol content and the relative amounts of the various sterols appear to be a function of species, diet, life stage and season (Connor et al., 2006; Koštál et al., 2013; Liland et al., 2017). For instance, in two species of sawflies (Hymenoptera) the predominant sterol was cholesterol (57%−73%), whereas in a third species 75% of the sterols present were in the form of 7-dehydrocholesterol (Svoboda et al., 1995). This indicates that large differences can exist in the primary storage form, even in closely related species. In contrast, in four cricket species provided with the same diet, the predominant sterol was cholesterol (83%−92%) at a concentration of 10−15 mg/g of fat (Tzompa-Sosa et al., 2021). High dietary levels of cholesterol increase the risk of atherosclerosis in humans and some monkey species, but the significance of dietary sterols for most insectivores is unclear. Corneal lipidosis and hypercholesterolemia has been reported in Cuban Tree Frogs (*Osteopilus septentrionalis* Duméril and Bibron) fed a diet of house crickets and occasionally day-old mice (Russell et al., 1990). In a later study Cuban tree frogs fed house cricket containing extremely high levels of cholesterol (1.7% dry matter basis) had a much higher incidence of corneal lipidosis than those fed house crickets containing 0.7% cholesterol or wild-caught Cuban tree frogs (Shilton et al., 2001).

18.3 Effects of insect size/life stage on nutrient composition

In general, the lipid content of wild-caught insects is approximately 30% for larvae and 20% for adults (Fast, 1970). Fat stores are usually greatest in the final larval stage prior to metamorphosis (Fast, 1970). This is more apparent for species which undergo complete metamorphosis (holometabolous species; for instance, yellow mealworms) than those with incomplete metamorphosis (hemimetabolous species; for instance, house crickets).

Hence, larvae of holometabolous species have a higher fat content than adults (Cookman et al., 1984; Finke, 2002; Lease and Wolf, 2011; Punzo, 2003). Yellow mealworms, for example, accumulate fat during larval growth (Finkel, 1948; McClements et al., 2003). These reserves are used as an energy source during metamorphosis, resulting in a lower fat content for adults and a thereby an increase in the relative content of protein and ash (Barker et al., 1998; Bernard and Allen, 1997; Downer and Matthews, 1976; Finke, 2002; Lease and Wolf, 2011; Oonincx and Dierenfeld, 2012; Ravzanaadii et al., 2012). A similar trend is seen in fruit flies (Church and Robertson, 1966), house flies (Pearincott, 1960) and black soldier flies (Liu et al., 2017). Furthermore, during metamorphosis the protein composition of yellow mealworms change; glycine, tryptophan and taurine content increase, while tyrosine content decreases (Finke, 2002; Ravzanaadii et al., 2012). The taurine content of holometabolous fruit flies (Massie et al., 1989), bertha armyworms (*Mamestra configurata*) (Bodnaryk, 1981) and yellow mealworms (Finke, 2002), increases after metamorphosis. This is consistent with the high concentration of taurine found in insect flight muscles (Whitton et al., 1987). The fly *Agria affinis* (Fallen) uses two-thirds of its fat reserve during pupation. This coincides with a relative decrease in C16:0, C16:1 and C18:1, and a relative increase in C18:2 and C22:1 (Barlow, 1965). Similarly, the fat content and fatty acid profile of black soldier fly larvae changes during development. Fat accumulates during the larval stage and then declines during pupation. Both C12:0 and C14:0 increase during larval growth while 16:0 and C18:1 decline (Liu et al., 2017; Giannetto et al., 2020a). Different changes in fat composition occur in silkworms. During their larval development, both fat content and composition change; the total fat content increases with relative increases of C16:0, C16:1, and C18:1 and decreases of C18:0 and C18:2. During pupation, C18:1 further increases while C16:0 continues to decrease. In the velvet bean caterpillar, total lipid content decreases during metamorphosis however C16:0 and C18:1 levels increase, while C18:3 levels decrease (Cookman et al., 1984). To what extent these changes are species-specific or constitute differences between Diptera and Lepidoptera needs further investigation. Similar to species preparing for

pupation, preparation for diapause or hibernation results in increased energy (fat) reserves (Ali and Ewiess, 1977; Downer and Matthews, 1976). In aquatic insects the concentration of C20:4 (arachidonic acid) and C20:5 decrease after the larval phase, when the insects leave the aquatic environment (Hanson et al., 1985; Sushchik et al., 2013).

Unlike holometabolous insects, hemimetabolous adults tend to have a higher fat content than nymphs (Lease and Wolf, 2011; Lipsitz and McFarlane, 1971). This holds true for some but not all studies conducted on migratory locusts (Oonincx et al., 2010; Oonincx and van der Poel, 2011). Probably the time of sampling, whether directly after adult emergence or later during adulthood, explains these differences since fat continues to accumulate after adult emergence (Beenakkers et al., 1985). Generally, flying and migratory insects accumulate more fat, which is used as an energy source for flight (Downer and Matthews, 1976). In this locust species, the dry matter content increases between the penultimate instar and adulthood (Oonincx and van der Poel, 2011). Interestingly, in both migratory and desert locusts (*Schistocerca gregaria* Forsskål) C18:3n3 comprises 9%−12% of the fatty acids in penultimate specimens, whereas this fatty acid is absent in adults (Oonincx et al., 2015). Similar to migratory locusts, the dry matter content of house crickets significantly increases (from 23% to 30%) during the penultimate stage of development (Roe et al., 1980). Adult house crickets have a higher protein and a lower fat content than small and medium nymphs (Finke, 2002; McClements et al., 2003). The amino acid composition seems to remain similar between nymphs and adults of this species (Finke, 2002). In a study of three cockroach species [Turkestan cockroaches, six-spotted cockroaches (*Eublaberus distanti* (Kirby)) and hissing cockroaches (*Gromphadorhina portentosa* (Schaum))], an increase in dry matter and crude protein content, and a concomitant decrease in fat content, was noted between small and medium specimens. The opposite change was noted when six-spotted roaches reached adulthood (Oonincx and Dierenfeld, 2012).

Besides size differences, certain gender differences seem apparent in insects, males often having a lower body weight than females, which might be caused by a lower fat content as mentioned previously (Ali and Ewiess, 1977; Hoffmann, 1973; Sonmez and Gulel, 2008).

18.4 Effects of insect diet on insect nutrient composition

Diet can have a significant effect on the nutrient composition of an insect. Since the entire insect is normally consumed, dietary effects described in the literature can partially be due to diet remaining in the gut which is discussed in more detail later in this chapter. The body composition of the insect itself can also be altered to a certain extent through the diet. For instance, higher water content of wheat bran (8.7 vs 6.7%) provided to yellow mealworm larvae increases their water content (64 vs 59%) (Machin, 1975).

For the macro nutrients, it seems that insect fat content is highly variable and that the relative content of protein and ash are subsequently affected. If an insect with a certain amount of protein increases its fat reserve, the percentage of protein thereby decreases (Oonincx and van der Poel, 2011; Simpson and Raubenheimer, 2001; St-Hilaire et al., 2007). This might be the reason why the protein content stays constant during adulthood, if a protein source is available and protein content decreases if only a carbohydrate source, which can be converted to fat, is available as was shown in Mediterranean fruit flies [*Ceratitis capitata* (Wiedemann)](Nestel et al., 2005). Although the percentage of protein can be altered through the diet, it seems unlikely that the amino acid composition is affected as mentioned previously.

As stated before, the crude fat content of insects can be altered by the diet provided. Two independent studies on fruit flies using the same artificial diet showed a similar nutrient composition. A third study on fruit flies found dissimilarities in fat and iron, which could indicate a strong dietary influence (Barker et al., 1998; Bernard and Allen, 1997; Oonincx and Dierenfeld, 2012). For house crickets, several studies are available on their chemical composition. Large differences in fat content have been reported ranging from 17% to 37% dry matter (Barker et al., 1998; Finke, 2002; Hatt et al., 2003; Oonincx et al., 2010). It seems likely that variation in fat content is a result of both the diet provided and the age of the cricket (Hatt et al., 2003). The protein and fat content of adult mealworm beetles can also be significantly affected by the protein and carbohydrate content of the larval diet (Rho and Lee, 2014).

Besides the plasticity of total fat content, large differences can be expected in the fatty acid composition when different diets are provided. In nonruminant production animals, short term provision of poly-unsaturated fatty acids increases the poly-unsaturated fatty acid content of the meat indicating that these fatty acids were incorporated in the body (Kouba and Mourot, 2011). Studies on insects indicate that the fatty acid composition of both larvae and adults tends to reflect the fatty acid composition of the diet provided (Cookman et al., 1984; Madariaga et al., 1971; Meneguz et al., 2018; Schaefer, 1968; St-Hilaire et al., 2007; Starčević et al., 2017; Rutaroa et al., 2018; van Broekhoven et al., 2015). The fatty acid composition of the insect however does not directly match that of the diet, suggesting selective accumulation or synthesis (Cookman et al., 1984; Hoc et al., 2020). For instance, in migratory locusts, the C18:1 and C18:3 content of the diet strongly relates to the fatty acid content of the locust fat body. However, for C18:2 this was

not the case, possibly due to poor absorption or due to conversion to C18:1 via saturation (Beenakkers and Scheres, 1971). While diet composition influences the fatty acid composition directly, indirect effects have also been reported. For instance a sufficient supply of C18:2, can be used to synthesize C20:4 and C20:5 (Hanson et al., 1983). Another prime example is the de novo synthesis of C12:0 in black soldier fly larvae (Hoc et al., 2020). Whereas C12:0 is the predominant fatty acid in this species, the total fatty profile can be greatly affected, for instance by supplying (C18:3n3) (Oonincx et al., 2019).

For many feeder insects, carrots are a well-accepted part of the diet or a means of providing moisture. In migratory locusts, the addition of carrots increases carotene and retinol (vitamin A) content (Oonincx and van der Poel, 2011). While the elevated carotene levels could be due to gut loading, it seems likely that the increased retinol content is due to the conversion of carotene to retinol. Likewise, when the medium of fruit fly larvae was enriched with carrot juice, larva, pupa and adults contained enhanced levels of both β-carotene and xanthophylls although the relative amounts varied between larva and adults (Giovannucci and Stephenson, 1999). Fruit flies can convert β-carotene to retinal, suggesting that more insect species have this ability (Von Lintig and Vogt, 2000). Great tits (*Parus major* L.) are able to distinguish carotenoid-enriched yellow mealworms from nonenriched counterparts and prefer the former (Senar et al., 2010).

18.5 Effects of environment on insect composition

A number of environmental factors, such as temperature, light and humidity, can affect growth, development and chemical composition of insects.

18.5.1 Temperature

Insects are poikilothermic, that is, cold-blooded; their body temperature depends to a large degree on the environmental temperature. Therefore within a range of temperatures suitable for the specific species, their metabolic rate and growth rate increases with higher temperatures (Akman Günduüz and Gülel, 2002; Ali and Ewiess, 1977; Ali et al., 2011; Angilletta et al., 2004; Krengel et al., 2012). A standard way of quantifying this is the relative difference when the temperature is increased by 10 degrees (Q10). For instance, in house crickets the development time is halved with a 10-degree increase in temperature (Roe et al., 1980). While growth rates are increased by higher temperatures, adult size generally seems to decrease (Akman Guünduüz and Gülel, 2002; Angilletta et al., 2004; Krengel et al., 2012) although the opposite effect was observed for both desert locusts and migratory locusts (Akman Guünduüz and Gülel, 2002; Beenakkers et al., 1971). Last instar female house crickets gain weight more quickly and more efficiently at 35°C than at 25°C, or 30°C (Roe et al., 1980, 1985). At higher temperatures, lipid content decreases during the latter half of this instar, but at 25°C carbohydrates are still converted to lipids. It seems that optimal growth occurs at 35°C, which is similar to the 34°C for the two-spotted field cricket (Hoffmann, 1973). However, for the latter species, mortality is also the highest at 34°C. Both growth rate and mortality are decreased at 27°C. For two-spotted field crickets temperature changes during rearing (alterations between 20°C and −1.5°C) versus constant temperatures (13°C) increase water content and decrease protein content (Hoffmann, 1973). Alternating temperatures around the optimal growth temperatures appear to increase protein and fat content in this species (Hoffmann, 1973). At low temperatures (13° and 20°C vs 27°C), fat content is higher, as are the proportions of unsaturated fatty acids. This seems a likely adaptation to the thermal regime. In the fly *Pseudosarcophaga affinis* (Fallén), higher proportions of saturated fatty acids increase heat tolerance (House et al., 1958). A comparative study on the seven-spotted [*Coccinella septempuctata* (L.)] and the Asian lady beetle [*Harmonia axyridis* (Pallas)] indicated that the first species accumulates more fat at elevated temperatures compared to normal temperatures (18° vs 21°C). Fat accumulation remains low under both conditions in the latter species. Females of the seven-spotted lady beetle have a higher fat content than males at normal temperatures, but a similar fat content at elevated temperatures. The carbohydrate and protein content of bean beetles [*Acanthoscelides obtectus* (Say)] is lower at 20°C than at 30°C, but lipid content is the same (Sonmez and Gulel, 2008).

18.5.2 Humidity

Humidity is normally expressed as relative humidity (RH), which is the relative amount of water that can be stored in the air at a certain temperature. Higher levels of humidity, within an appropriate range, seem to allow for more rapid development (Ali et al., 2011; Han et al., 2008; Nedvěd and Kalushkov, 2012). However, unlike in the case of increasing temperatures, this increase in growth rate does not seem to lead to a lower body mass in adults. For instance, the

body mass of the pine caterpillar, *Dendrolimus tabulaeformis* (Tsai et Liu) increases with higher humidity during their larval stages (20%−100%) (Han et al., 2008).

A comparative study on the effects of humidity and temperature on locust species (*L. migratoria*, and *S. gregaria*) indicated development is possible between 35% and 80% RH, with 60%−65% being optimal, and increasing temperatures require a higher RH (Hamilton, 1936). Similarly, pine caterpillars require an RH above 40% for development, while the optimal RH for maximal body mass seems to be around 80%. During diapause, this species absorbs water from the substrate, thereby increasing its live weight (Han et al., 2008). Fasting mealworms are capable of absorbing water vapor if RH is above 88% RH (Fraenkel, 1950; Machin, 1975). RH also has an indirect effect through the feed provided as well. If the feed provided to yellow mealworms is in equilibrium with 70% RH, it allows for rapid growth (Fraenkel, 1950). High humidity increases the dry matter weight gain of yellow mealworms postexposure (Machin, 1975). However long-term exposure to high humidity ($>85\%$) results in higher larval mortality, probably due to excessive hydration (Machin, 1975). Other problems are likely to occur at high RH, for instance, the development of fungi and/or mites (Machin, 1975). The optimal growth rate for mealworms at 25°C is attained at 70% RH (Fraenkel, 1950; Machin, 1975).

As indicated before, the optimal RH also relates to temperature, although other variables can play a role as well. The Asian lady beetle tends to grow larger at higher humidities (between 30% and 90%) if fed with the aphid *Acyrthosiphon pisum* (Harris) but not when fed on frozen eggs of *Ephestia kuehniella* (Zeller) (Nedvěd and Kalushkov, 2012).

It seems that the body weight and development rate of insects are higher at the top of their RH range. The moisture content of insects also seems to increase at a higher RH. However, little is known about how body composition is affected by changes in RH.

18.5.3 Photoperiod

The effect of photoperiod (daily exposure time to light) on insect composition has not been studied in detail; most studies have focused on behavioral effects, fecundity and body weight. For certain locust species, long photoperiods (up to 24 hours) increase their growth rate, possibly as a result of increased food intake. At a constant temperature, Asian lady beetles tend to develop more quickly with 16 hours of light compared to 12 hours of light, but the adult weight was similar (Berkvens et al., 2008). This seems likely for these diurnal species, but the green stink bug [*Nezara viridula* (L.)] that mainly feeds at night, has an increase in the rate of development with an increased photoperiod (10 vs 14 hours) resulting in an increase in body weight when reaching, and during adulthood (Ali and Ewiess, 1977; Shearer and Jones, 1996). Short photoperiods can induce diapause, while longer photoperiods are more likely to induce reproduction. It seems that for this species, more energy is accumulated when preparing for reproduction than for diapause, which would explain the higher fat content. However, little is known about the influence of photoperiod on the chemical composition. The protein content of adults of the giant wax moth increases more rapidly if kept in constant light, compared to constant darkness (Koc and Gulel, 2008). The effects of photoperiod on the nutrient profile of insects mostly seem to be indirect, acting through other processes, such as preparation for reproduction or diapause or concomitant changes in temperature.

18.6 Nutrient requirements of insectivores including nutrient availability

It is difficult to compare the nutritive value of insects as a group to the dietary requirements of insectivores because of the large number of insect species and the large differences in nutrient content between those species (Barker et al., 1998; Bukkens, 1997; Finke, 2002, 2013). A proper evaluation of a food/insect requires it to be evaluated in the broader context of a complete diet, made up of a number of different foods/insects.

18.6.1 Availability and digestibility

It has been suggested that insects might be poorly digestible because of their sclerotized, chitin-rich, exoskeletons. Due to the recent interest in the use of insects as feed ingredients for production animals, there is now an abundance of data on the protein digestibility of various insect meals. Of note, however, is that the insects used in these feeding trials are heated to remove excess moisture, finely ground and in some cases defatted and then incorporated into mixed diets. Hence, the application of these values to that of raw whole insects is unclear. That said, in most studies, the protein and amino acid digestibility of insect meals equals, or surpasses the more conventional protein sources. This includes studies when insects meals are fed to rats (Dreyer and Wehmeyer, 1982; Xia et al., 2012), pigs (Biasato et al., 2019; Jin

et al., 2016; Yoo et al., 2018), shrimp (Panini et al., 2017), various species of poultry (Benzertiha et al., 2019; Bovera et al., 2016; De Marco et al., 2015; Hall et al., 2018; Hwangbo et al., 2009; Pieterse and Pretorius, 2013; Schiavone et al., 2017; Wang et al., 2007; Woods et al., 2019), and fish (Alegbeleye et al., 2012; Basto et al., 2020; Belforti et al., 2015; Belghit et al., 2019; Chemello et al., 2020; Dumas et al., 2018; Fontes et al., 2019; Gasco et al., 2016; Magalhaes et al., 2017; Piccolo et al., 2017; Renna et al., 2017).

There are some reports on whole dried insects fed to rats in purified diets. Unlike the feeding trials mentioned previously, in these studies the material was freeze-dried to remove excess moisture and the dried insects were the only source of protein in the diet. These studies showed that the apparent protein digestibility of ground, freeze-dried yellow mealworm larvae was 75%−91% when fed to rats (Goulet et al., 1978; Jensen et al., 2019; Poelaert et al., 2018), while for ground freeze-dried house crickets apparent protein digestibility was 78%. (Poelaert et al., 2018). The digestibility of these insects was only slightly lower than for casein (88%−93%), a highly digestible milk protein (Goulet et al., 1978; Poelaert et al., 2018). Similarly, Jensen et al. (2019) report a high true protein digestibility of freeze-dried yellow mealworms (92%) and lesser mealworms (94%) when fed to rats.

There are two studies regarding the protein digestibility of raw whole insects when fed to insectivores. Crude protein digestibility of black soldier fly larvae fed to mountain chicken frogs [*Leptodactylus fallax* (Müller)] was low (44%) for live larvae, but the digestibility increased to 77% when the larvae were mashed (Dierenfeld and King, 2008). The crude protein digestibility of intact house crickets when fed to these frogs was 95% (Dierenfeld and King, 2008). The lower protein digestibility of intact versus mashed larvae could be due to the protective properties of the mineralized exoskeleton, inhibiting the digestives enzyme's access to the more digestible nutrients. The protein digestibility of black soldier fly larvae in leopard geckos (*Eublepharis macularius*) was 81% (Boykin and Mitchell, 2021). These geckos are more likely than mountain chicken frogs to chew on their prey, thereby mechanically damaging the exoskeleton.

In general, protein digestibility of insects is high and the variability reported in the literature is likely a result of differences in how the insects were prepared and the proportion of amino acids used for sclerotization. Insects with a large proportion of their amino acids in a cuticular protein, complexed with chitin, might have a lower protein digestibility than those that do not (Finke, 2007).

Besides protein digestibility some information on mineral digestibility is available. Concerns have been raised about calcium availability from black soldier fly larvae, especially for insectivores that swallow their prey whole. When fed to mountain chicken frogs the calcium and phosphorus digestibility of whole black soldier fly larvae was only 44% and 51% respectively compared to 88% and 91% for larvae that had been "mashed" (Dierenfeld and King, 2008). For comparison when fed to mountain chicken frogs the calcium and phosphorus digestibility for crickets was 84% and 93%, respectively (Dierenfeld and King, 2008). Similarly, the calcium and phosphorus digestibility of whole and "pierced" black soldier fly larvae when fed to leopard geckos was 43% and 45%, respectively (Boykin et al., 2020). Even with a low availability, the high calcium concentration in these larvae should provide adequate amounts of calcium for most insectivores.

18.7 Enhancing the nutrient composition of insects as food for insectivores

While the exact nutrient requirements for most insectivores are unknown, certain nutrient deficiencies are known to occur regularly in captive insectivores. The three most commonly reported are calcium, vitamins A, and D deficiencies. Insectivore diets can be enhanced by increasing the concentration of nutrients which are expected to be present at too low of a level in the insect. There are three main methods to do so; gut-loading, dusting or feeding the insect an enriched diet during growth. The goal of all these methods is to increase the intake of selected nutrients by the insectivore. While this is valid for a certain range, one must be careful that this range is not surpassed, resulting in adverse effects. For instance, over-supplementation with calcium can decrease the absorption of other minerals, leading to secondary trace mineral deficiencies. Likewise, over-supplementation of the fat-soluble vitamins, in particular vitamins A and D, can cause toxicity (Bender, 2002). A difference in size (developmental stage) of the dusted or gut loaded insect leads to differences in a surface: volume and gut size: volume ratios. Hence, smaller insects have a larger relative surface area to which dust can adhere. The same powder used for pinhead or adult house crickets, therefore, has a greater effect on the smaller specimens (Sullivan et al., 2009). Similarly, for gut-loaded insects size differences could lead to differences in nutrient delivery of smaller versus larger insects (Finke, et al., 2005).

18.7.1 Gut loading

Gut loading is the term used for the provision of a special diet to insects, shortly before the insects are consumed. These diets contain high levels of the desired nutrient(s), which are present in the insect gut when the insect is

consumed. Therefore, the insectivore's intake of those nutrients increases. Due to the nature of gut loading, it is suitable for almost all nutrients as long as the diet is palatable to the insect and the diet contains sufficient quantities of the desired nutrient(s) (Hunt-Coslik et al., 2009).

Most research on the effects of gut loading has focused on increasing calcium content. High calcium gut load diets containing 4%−9% calcium, typically from calcium carbonate, are effective in increasing the calcium content of wax moth larvae, house crickets, yellow mealworm larvae, superworm larvae and silkworm larvae (Allen and Oftedal, 1989; Anderson, 2000; Finke, 2003; Finke, et al., 2005; Klasing et al., 2000; Latney et al., 2017; Strzeleqicz et al., 1985). Chemical analysis of the diet is recommended to verify the true calcium content of commercially available gut-loading diets (Finke, et al., 2004, 2005). The calcium from yellow mealworms gut loaded with a high calcium diet was readily available to growing chicks showing the usefulness of this method (Klasing et al., 2000). The optimal gut loading time seems to vary slightly. This is likely a result of the insect species, diet palatability and environmental conditions (temperature, light and humidity). In general, however, gut loading for 24−72 hours appears to result in similar levels of nutrients in the intact insect. When gut-loading diets are fed for longer periods of time adverse effects on the viability of the insects can occur (Klasing et al., 2000). In yellow mealworms a gut loading period of 24 hours increased calcium levels and improved calcium to phosphorus ratios, and extending this period to 48 or 72 hours, resulted in a further increase (Klasing et al., 2000; Anderson, 2000). For house crickets, a period of 48 hours seems sufficient to attain a significant increase in calcium to phosphorus ratios, while extending this to 72 hours does not affect this ratio (Anderson, 2000). However, other studies report the highest calcium content after 1 day compared to 2, 3, or 7 days (Dikeman et al., 2007). Dietary calcium to phosphorus ratio of 1:1 to 2:1 is considered optimal for most species. Offering certain gut loading diets longer than two days reduces the initially increased calcium levels (Hunt-Coslik et al., 2009), which could be an effect of diet palatability (McComb, 2010).

In addition, the physical form of the nutrient and the presence of other nutrients affecting palatability should be considered when designing a gut loading diet (Anderson, 2000; Finke, et al., 2005). The addition of poly-unsaturated fatty acids to the gut loading diet has been suggested for insectivores from temperate climates which would be likely to encounter insects with relatively high poly-unsaturated fatty acids concentrations (Li et al., 2009).

Gut-loading can also increase the vitamin A content of crickets, yellow mealworm larvae, black soldier fly larvae and silkworm larvae (Boykin and Mitchell, 2021; Finke, 2003). Insect retinol levels increased either linearly or curvilinearly with increasing dietary levels and at the highest dietary level insects contained sufficient vitamin A to meet the anticipated requirements of most insectivores. When gut-loading insects with vitamin A it is critical that the physical characteristics of the supplement, such as particle size and hardness, allow for adequate consumption by the insect (Livingston et al., 2014).

A study in which yellow mealworms were gut loaded with chicken starter feed, increased their vitamin D content to 132 IU/kg dry matter, while undetectable levels were present in mealworms provided a wheat bran diet (Klasing et al., 2000). A simple way of providing extra carotenoids to insectivores, is the provision of carrots or other fruits or vegetables during the last 24 hours before feeding the insects to the insectivores. The amount of carotenoids that accumulate differs per insect species. The field cricket, for instance, accumulates more carotenoids on a high carotenoid diet than the house cricket, or the banded cricket [*Gryllodes sigillatus* (Walker)] (Ogilvy et al., 2012).

Gut-loading can also increase the levels of omega-3 fatty acids in black soldier fly larvae (Barroso et al., 2017). Larvae fed a control diet with 40% added fish meal had increased levels of the fatty acids 20:5 and 22:6. The omega-3 fatty acid level in the larvae increased rapidly and appeared to reach a plateau after about 3 hours suggesting the increases were primarily a result of gut-loading and not accumulation in the tissues of the insect.

18.7.2 Dusting

Dusting is a term used for coating an insect with a fine powder containing the desired nutrients, such that the powder adheres to the outside of the insect. When the insect is eaten, this powder is also ingested. Little scientific data exist on the nutritional effects of this method. One study in which house crickets were dusted with calcium carbonate powder, the calcium to phosphorus ratio increased from 1:5.7 to 5.3:1 and the digestibility of these minerals was high (84% and 94%, respectively) (Dierenfeld and King, 2008). Whereas dusting can be an effective method for animals which immediately consume their prey, the effectiveness decreases with the time between dusting and consumption (Trusk and Crissey, 1987). House crickets, for instance, can groom off half of the adhering powder in 150 seconds (Li et al., 2009). Another study, quantifying the amount of calcium carbonate adhering to insects after dusting indicated that house cricket weight increased by 13.4% directly after, whereas this decreased to 8.7% after 15 minutes, and 4.5% after 30 minutes (Oonincx et al., 2020). For two locust species the starting weight increased by 5.5%−5.6% due to dusting

and halved after 15 minutes, and then again halved after 30 minutes. Another downside of dusting is that it is difficult to quantify the amount of dust adhering to the insect, and hence the dosage provided to the insectivore. This would depend on the physical characteristics of both the dust and the insect exoskeleton, and the relative surface area of the insect. The weight of giant wax moth larvae, yellow mealworm larvae and house crickets increased by 0.8%−6.3% when dusted with two types of calcium carbonate dusts (Winn et al., 2003). For aquatic insectivores, this way of enhancing the nutrient content is obviously unsuitable. Both the composition and the method of providing the dust or the gut load diet can affect the weight gain of insectivores, as was shown for the Wyoming toad (*Bufo baxteri* Porter) (Li et al., 2009). In that specific case, a lower weight gain coincided with decreased consumption, which in turn might be caused by decreased palatability due to the vitamin powder (Li et al., 2009). A study of Puerto Rican crested toads (*Peltophryne lemur* (Cope)), compared the direct oral application of vitamin A with gut loading and dusting in house crickets. Retinol blood values were higher in toads offered dusted crickets (McComb, 2010). Why the other methods were less effective is unclear. Possibly, retinol is better absorbed when ingested with food, as dietary fat can enhance the absorption of fat-soluble nutrients such as retinol. Hence, the fat in the crickets may have increased retinol absorption compared to direct oral supplementation.

18.7.3 Feeding nutrient enhanced diets during growth

It is now well established that select nutrient concentrations in insects can be altered by manipulating the content of the diet. This is different from gut-loading in that the nutrient changes are not a result of the residual food in the gastrointestinal tract, but rather the nutrients are incorporated into the tissue of the insect. While not effective for all nutrients and for all insect species, nutrients that can be significantly altered using this technique in some species include fatty acids, calcium, carotenoids, and vitamin E.

18.7.3.1 Fatty acids

Early research indicated that the fatty acid composition of insects partially reflects their diet (Cookman et al., 1984; Madariaga et al., 1971; Schaefer, 1968). More recently the fatty acid composition of a number of insects, commonly fed to captive insectivores, has been modified to increase either the levels of omega-6 or omega-3 fatty acids. This includes enhancing the omega-3 content of yellow mealworms (Dreassi et al., 2017; Fasel et al., 2017; Finke, 2015a, Oonincx et al., 2019; van Broekhoven et al., 2015), lesser mealworms (Oonincx et al., 2019), house crickets (Finke, 2015a, Komprda et al., 2013; Oonincx et al., 2019), Jamaican field cricket (*Gryllus assimilis*) (Starčević et al., 2017), superworms (Finke, 2015a), waxworms (Finke, 2015a), silkworms (Chieco et al., 2019) and black soldier fly larvae (Ewald et al., 2020; Meneguz et al., 2018; Oonincx et al., 2019; Spranghers et al., 2017; St-Hilaire et al., 2007). The amount of omega-3 fatty acids and the omega-6 to omega-3 ratio have been implicated as being beneficial in many species due to their role in cell membrane function, gene expression, and inflammation (Schmitz and Ecker, 2008). It is unclear if they might confer similar benefits to insectivores.

18.7.3.2 Calcium

Most insects have a low calcium content. However, as mentioned previously, larvae of both the face fly and the black soldier fly can contain significant amounts of calcium. For black soldier fly larvae the calcium content depends on the diet, with calcium levels ranging from 0.84% to 8.29% (dry matter), with calcium to phosphorus ratios of 1.1:1 to 4.7:1 (Liland et al., 2017; Schmidt et al., 2019; Tschirner and Simon, 2015). These data include both calcium in the insect body exoskeleton and dietary calcium residing in the gastrointestinal tract. Calcium levels in black soldier fly prepupae, which have emptied their gut prior to pupation ranged from 0.12% to 6.61% (dry matter) with calcium to phosphorus ratios of 0.3:1 to 14.9:1 (Spranghers et al., 2017; Wang et al., 2020).

18.7.3.3 Carotenoids

Insect diets rich in carotenoids increase the carotenoid levels in the insect's tissues. However, the amounts retained depend on the carotenoid in question, the dietary levels, and the insect species. Feeding a diet rich in β-carotene increases β-carotene levels in house crickets, migratory locusts, mealworm larvae and superworm larvae (Finke, 2015a; Oonincx and van der Poel, 2011). In contrast, a diet containing β-carotene did not result in any β-carotene retention in blow flies (*Calliphora*) (Vogt and Kirschfeld, 1984), fruit flies (Giovannucci and Stephenson, 1999), and waxworm larvae (Finke, 2015b) and only low levels in silkworm larvae (Chieco et al., 2019). This difference is likely a result of the chromophore the insect uses for vision. While some insects like dragonflies use both retinal and 3-hydroxy retinal as their chromophore

most other insects use either retinal or 3-hydroxy retinal (Smith and Goldsmith, 1990). Orthoptera (including crickets and locusts), Coleoptera (including both mealworms and superworms) and Blattodea (roaches) use retinal which is synthesized by cleaving one molecule of β-carotene into two molecules of retinal. In contrast Lepidoptera (including waxworms, butterworms, silkworms and hornworms) and Diptera (including fruit flies and black solider flies) use 3-hydroxy retinal as their chromophore which is synthesized from zeaxanthin. For insects that use 3-hydroxy retinal as their chromophore, dietary β-carotene is first converted to zeaxanthin and then to 3-hydroxy retinal (Giovannucci and Stephenson, 1999; Voolstra et al., 2010). As such it is important to know if the insect being used as food uses retinal or 3-hydroxyretinal as their chromophore since β-carotene has vitamin A activity while zeaxanthin does not.

18.7.3.4 Vitamin E

Increased dietary vitamin E increases the vitamin E content of insects. This was shown for both black soldier fly larvae and mealworms. However, as those larvae were not fasted prior to analysis, at least part of this increase was due to dietary vitamin E remaining in their gastrointestinal tract (Liland et al., 2017; Pennino et al., 1991). Data for house crickets, mealworms, superworms and waxworms, which were fasted prior to analysis, indicates that vitamin E levels also increase in their tissues when fed diets containing high levels of vitamin E (Finke, 2015a). This is similar to swine, where high levels of dietary vitamin E result in enhanced levels in various tissues (Asghar et al., 1991).

18.8 Other considerations

18.8.1 Pathogens/parasites

There is little information regarding commercial feeder insects as a source of pathogens. House crickets from five commercial suppliers in the United States were shown to be free of Oxyurids/pinworms but little other information is available regarding insects as a source of parasites for insectivores (Klarsfeld and Mitchell, 2005). One exception is a study in which black soldier fly larvae were provided with either coccidian parasites [*Eimeria tenella* (Tyzzer) or *Eimeria nieschulzi* (Dieben)] or nematode [*Ascaris suum* (Goeze)] eggs (Muller et al., 2019). After 10 days all three parasites were recovered from the outside of the black soldier fly larvae and prepupae. Moreover, these parasites were also recovered from the intestines of the larvae and, in part, from the intestines of the prepupae indicating that they could vector these parasites.

Several studies have been done on the vectoring capacity of insects regarding pathogenic bacteria. The lesser mealworm can vector *Salmonella spp*. with both larvae and adults carrying *Salmonella spp*. both externally and internally (Crippen et al., 2009). Additionally, *Salmonella* was detected in newly emerged adults from infected larvae suggesting some bacteria are carried through metamorphosis (Crippen et al., 2012). Moreover, while black soldier fly larvae fed chicken manure inoculated with *Salmonella enterica* (Kauffmann and Edwards) Le Minor & Popoff (6.9 log CFU/g) reduced the concentration of *S. enterica* in the manure, populations were still present at 3.3 log CFU/g after three, and at 2.2 log CFU/g after six days (Erickson et al., 2004). Thus, it seems likely that these, and other insect species, could vector *Salmonella spp*. and other pathogenic microorganisms. Therefore, these species and others should be obtained from qualified suppliers, fed appropriately, and maintained hygienically, to minimize potential transmission.

18.8.2 Heavy metals

Insect species differ in how they cope with heavy metals. Whereas differences between species exist, cadmium accumulation is consistently suggested for several species of flies, including houseflies (Charlton et al., 2015), fruit flies (Maroni and Watson, 1985), flesh flies (*Sarcophaga peregrina* Robineau-Desvoidy) (Gao et al., 2017) and black soldier fly larvae (Diener et al., 2015; Gao et al., 2017; Purschke et al., 2017; van der Fels-Klerx et al., 2016; Wu et al., 2020). In contrast, mercury, chromium, and arsenic are largely excreted by black soldier fly larvae (Biancarosa et al., 2018; Diener et al., 2015; Gao et al., 2017; van der Fels-Klerx et al., 2016). It seems that lead and zinc accumulate in the outer layer of the larval exoskeleton when the larvae are exposed to higher dietary concentrations (Diener et al., 2015). For another group of commonly used feed insects, the Orthoptera, cadmium accumulation also seems to be a risk factor. This was shown for seven different acridid species and in the Jamaican field cricket (Bednarska et al., 2015; Devkota and Schmidt, 2000a, 2000b; Zhang et al., 2012). However, a study with house crickets indicates that cadmium from a pulse dose can be rapidly excreted (Van Hook and Yates, 1975). In the yellow mealworm dietary cadmium does not seem to accumulate, however dietary arsenic does (van der Fels-Klerx et al., 2016; Vijver et al., 2003). Essential elements such as copper, zinc and iron seem carefully regulated in the yellow mealworm (Keil et al., 2020; Vijver et al.,

2003). This seems to be a general trend for essential elements in insects, for instance, zinc is carefully regulated in the Jamaican field cricket (Bednarska et al., 2015), as is zinc in larvae of the black soldier fly (Diener et al., 2015). The latter species loses excess zinc via ecdysis. A similar method seems to be used by yellow mealworms which stores cadmium in their exoskeleton and can thereby decrease cadmium concentrations via molting and metamorphosis (Lindqvist and Block, 1995; Lindqvist and Block, 1997). Increasing levels of dietary zinc aid in reducing cadmium accumulation (Keil et al., 2020). Such mechanisms help the insect cope with exposure to heavy metals. Still, the presence of heavy metals in the insect's diet should be avoided as these can transfer to the consuming insectivore, be it via storage in the insect body, or via the food remaining in its gastrointestinal tract.

18.8.3 Mycotoxins

Ingredients commonly used in commercial insect diets can become contaminated with various species of molds which can produce mycotoxins. Several studies have been done to determine the effects on commonly reared insect species. For the yellow mealworm, the following mycotoxins were investigated: aflatoxin B1 (Bosch et al., 2017) deoxynivalenol (Ochoa Sanabria et al., 2019; van Broekhoven et al., 2017), and zealerone (Niermans et al., 2019). A similar list is available for the lesser mealworm: aflatoxin B1 (Camenzuli et al., 2018), deoxynivalenol (Camenzuli et al., 2018 Leni et al., 2019), fumonisin 1 and 2 (Leni et al., 2019), ochratoxin A (Camenzuli et al., 2018), and zearalenone (Camenzuli et al., 2018; Leni et al., 2019). The black soldier fly has also been extensively tested with aflatoxin B1 (Bosch et al., 2017; Camenzuli et al., 2018; Purschke et al., 2017), aflatoxin B2 (Purschke et al., 2017) deoxynivalenol (Leni et al., 2019; Camenzuli et al., 2018; Purschke et al., 2017), fumonisin 1 and 2 (Leni et al., 2019), ochratoxin A (Camenzuli et al., 2018; Purschke et al., 2017), and zearalenone (Camenzuli et al., 2018; Leni et al., 2019 Purschke et al., 2017). Unfortunately, quantitative studies on Orthoptera are currently not available, although these are to be expected in the coming years.

The results obtained in the aforementioned studies are remarkably consistent: (1) mycotoxin exposed insects developed and grew normally; (2) mycotoxins do not accumulate in the insect body; (3) low concentrations are sometimes detected, probably due to contaminated feed present in the gut; (4) mycotoxins are catabolized by the insect, but generally not to the better known and often also toxic metabolites. As there is the possibility that metabolites that have not been identified and detected in these insects are toxic, further insights into the exact pathways are needed. For now, however, there seems to be little risk of mycotoxins for insectivores.

18.8.4 Other toxins

It is well known that many species of insects sequester toxic compounds from their diet making them unpalatable or even toxic to certain insectivores. In the wild, these species are generally brightly colored (aposomatic) to warn potential predators of the consequences of feeding on these species. Monarch butterflies (*Danaus plexippus* L.) and milkweed bugs (*Oncopeltus fasciatus* Dallas) are but a few of the many species that sequester toxins from their feed (Berebaum and Miliczky, 1984; Brower, 1969). Since most captive-raised feeder insects are fed diets containing commercial feed ingredients also used for domestic animals, it seems unlikely that they would accumulate few if any toxins from their diet as long as the diet was properly made and stored.

Almost nothing is known regarding the potential antinutritional properties of insects except for thiaminase. Thiaminase is an enzyme that when ingested splits thiamine (vitamin B_1) effectively destroying its vitamin properties. While thiaminases are typically associated with certain species of fish (Spitzer et al., 1941) it has been reported in both domesticated silkworm larvae (*B. mori*) and African silkworm pupae (*Anaphe* spp), although the levels found in domesticated silkworm larvae were only one-third of those found in African silkworm pupae (Nishimune et al., 2000; Watanabe et al., 2001). These authors noted that in addition to thiamine, pyridoxine, taurine, and possibly other nutrients could also serve as a substrate for this enzyme. The consumption of *Anaphe* pupae has been associated with seasonal ataxia in humans in Nigeria, presumably due to thiamine deficiency (Adamolekun, 1993; Adamolekun et al., 1997). The extent to which thiaminases are found in other species of insects, and their potential effect on insectivores, is currently unknown.

18.8.5 Uric acid

Uric acid is produced by most insect species as a nitrogenous waste product from protein and purine metabolism. Some species, most notably cockroaches, store significant amounts of this nitrogen source in their fat body where bacterial endosymbionts can use it for amino acid synthesis (Patino-Navarrete et al., 2014). The amount of uric acid stored by

cockroaches depends on the species, age, sex, and most importantly diet (Mullins and Cochran, 1975a, 1975b). Uric acid levels in 23 cockroach species, determined with a uricase-spectrophotometric method, were up to 30% of the roach's dry weight, with an average value of 12.8% (Mullins and Cochran, 1976). Twenty of these species were fed commercial dog food containing 25% protein, which could partially explain these high levels. In another study, using the same method, house crickets contained only 2.6% uric acid on a dry matter basis (Cochran, 1976). More recently, uric acid concentrations measured by reverse-phase HPLC were between 4.8% and 7.0% dry matter for discoid cockroaches (*Blaberus discoidalis* Serville), and between 1.0% and 1.2% dry matter for house crickets (Sabolova et al., 2021). Whereas the reported values were lower, the contrast between these species was similar. Desert locusts contained only 0.44%−0.75% of their weight (dry matter) as uric acid. In some animal species a build-up of uric acid in the body, can lead to gout (Mader, 2006). Hence, insectivores that may be prone to develop gout should primarily be offered insects with low uric acid contents.

18.9 Conclusions

Insects are a good source of many nutrients although for most nutrients the values vary widely depending on the insect species, the life stage, the diet and the conditions in which they are raised. In general, most species are good sources of amino acids, fatty acids, most minerals and most B vitamins. Based on analysis of feeder insects and reports of nutrient deficiencies in captive insectivores the nutrients of concern in a captive insectivore feeding program are calcium, and fat-soluble vitamins A, D and E. As such, captive insectivores should be fed a mix of invertebrates that have been dusted, gut-loaded or fed nutrient enhanced diets during growth to provide a wide range of nutrient intakes to reduce the risk of nutrient deficiencies.

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Chapter 19

Production of solitary bees for pollination in the United States

Stephen S. Peterson[1] and Derek R. Artz[2]

[1]AgPollen LLC, Visalia, CA, United States, [2]US Department of Agriculture, Agricultural Research Service (USDA-ARS), Pollinating Insects Research Unit, Logan, UT, United States

19.1 Introduction

Many of our most valuable crops require an insect, usually a bee, for pollination. Bees pollinate some 400 crops worldwide and 130 in the United States (O'Toole, 2008). In addition, agriculture is becoming more dependent on the services of bees because the proportion of crops that require insect pollination has increased in recent years (Aizen et al., 2008). When a managed pollinator is provided, most often it is the familiar honey bee, *Apis mellifera* L., which is employed to do the job. For centuries, the honey bee was cultured for its production of honey and wax, and, more recently, intensive monoculture crop cultivation has made providing bees for a pollination fee an important aspect of beekeeping. Europeans brought the honey bee everywhere they settled, and it is now employed as a crop pollinator on every continent except Antarctica. But the ubiquitous honey bee is just one of about 16,000 described species of bees in 1200 genera worldwide (Michener, 2000). North America alone has 3800 species, of which 21 are introduced (Cane, 2003). Bees are most diverse in the warm-temperate, drier parts of the world; for example, California has 1985 species (Michener, 2000). The bees range from specialists that visit only one species of plant to generalists like the honey bee, and from solitary to eusocial, again like the honey bee. They are grouped into seven major families (Michener, 2000). The most familiar are the Halictidae (sweat bees), Megachilidae (leafcutting bees and mason bees), and Apidae (carpenter bees, bumble bees, and honey bees). Many of the solitary species visit crop plants, and some are now managed for their pollination services. The value of insect-pollinated crops in the United States was estimated to be $15.1 billion in 2009, $3.4 billion of which was attributed to non-*Apis* bees (Calderone, 2012).

Each bee species has its requirements for floral and nesting resources. Many bee species nest in the ground, which does not preclude them from being managed as a pollinator (e.g., *Nomia melanderi* Cockerell), but does present difficulties when it comes to moving them to a crop. Those species that nest above ground, or will accept structures above ground, are much easier to manipulate. Many of the Megachilidae nest in beetle borings in dead trees in the wild and these can be mimicked to provide a place for them to nest. Other Megachilids nest in crevices in embankments or use cavities in plant stems, and many of these will also accept artificial nest tunnels.

In this chapter, we will focus on the production of three species of solitary bees that have been employed as managed pollinators in the United States: the alfalfa leafcutting bee, *Megachile rotundata* F.; the alkali bee, *N. melanderi*; and the blue orchard bee, *Osmia lignaria* Say.

19.2 The alfalfa leafcutting bee

M. rotundata (Fig. 19.1) is native to Europe and arrived in North America in 1930 (Free, 1993). Nests of wintering prepupae (the inactive larval stage prior to the pupal stage) were probably inadvertently transported in wooden shipping materials. It gradually spread across the continent and reached the western United States in the 1950s. Stephen and Torchio (1961) observed this bee readily visiting and collecting alfalfa pollen. Honey bees were used for alfalfa seed production at that time, but Stephen (1955) observed that most honey bees would not collect pollen from alfalfa, instead approaching the flower from the side to gain access to the nectar. The reproductive structures of the alfalfa flower pop

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FIGURE 19.1 The alfalfa leafcutting bee, *Megachile rotundata*, on an alfalfa flower.

out explosively when a bee probes them, and honey bees apparently learn to avoid this triggering. The alfalfa leafcutting bee is undeterred by the alfalfa flower's mechanics, tripping 81% of flowers when allowed a single visit (Cane, 2002), and readily accepts artificial nesting sites (Stephen, 1961). Stephen (2003) noted that the North American and European *M. rotundata* are different in several ways that make the North American bee a better candidate for domestication. The accidental introduction must have created a genetic bottleneck resulting in a more gregarious population that flies at lower temperatures, completes nests faster, collects pollen from fewer hosts, collects more pollen, and is multivoltine rather than univoltine (Stephen, 2003; see citations within).

Alfalfa seed yield improved greatly where alfalfa leafcutting bees were employed. For example, in Idaho, yields were 110−220 kg/ha prior to the arrival of the alfalfa leafcutting bee, but yields climbed to 280−560 kg/ha after 1956, when the leafcutting bees became commonplace (Olmstead and Wooten, 1987). Mass rearing of this bee involves wintering the cocoons in cold storage, followed by incubation for about 4 weeks and release in the field. Cavities in wood, polystyrene, or paper tubes must be provided for nesting, with some protection from rain and direct sun. The females require an abundant source of pollen and nectar for 3−4 weeks for adequate reproduction. The cocoons may be left in their nesting substrate or stripped out, depending on the management system.

The alfalfa leafcutting bee usually has a single generation per year in northern latitudes. It is a relatively small bee of 7−9 mm in length, and it overwinters as a prepupa in its cocoon. In the spring, as temperatures warm, it continues its development through the pupal and adult stages and then chews out of its cocoon and nest. Females mate once (although males can mate more than once) and then search for a suitable place to nest. The female holds the sperm in a sac-like organ called the spermatheca, and as in all Hymenoptera, unfertilized eggs become males and fertilized eggs become females (arrhenotoky). Once a female has selected a cavity for nesting, she lines it with 14−16 cut leaf pieces (Richards, 1984a). Soft leaves or flower petals are preferred, and in alfalfa, leaves are used to line nests (Horne, 1995). A female can carry a leaf piece that is 17% of her body weight, which averages 35 mg (Klostermeyer et al., 1973). Leaves are carefully arranged to overlap, and the edges are chewed to make them adhere together. Next, the bee goes to work gathering pollen, which it packs onto the thick layer of hairs (the scopa) on its abdomen. The maximum pollen load size is about 23% of the bee's body weight. After arriving back at her nest, the female will turn around and back into the hole, removing the pollen with her hind legs. Nectar is regurgitated and added to the pollen provision to create a doughy mass. Females will make 15−27 provisioning trips and take about 5 hours, under ideal conditions, to provision a single cell (Richards, 1984a).

When the pollen provision reaches a suitable size, the female deposits an egg on it. The cell is then sealed off with leaf disks, and the process starts over. Depending on the length, a female will construct 8 to 12 cells in a cavity (Richards, 1984a). Female eggs are deposited at the back of the hole, and males toward the entrance. The ratio of males to females has been reported as 2:1 (Richards, 1984a), but recent studies show that the ratio of males to females in commercial populations in the United States is close to 1:1 (Pitts-Singer and James, 2005). The larvae have five instars, although the first instar remains inside the egg (Trostle and Torchio, 1994). After the eggs hatch, 14.5 days are required to complete larval development at 23°C (Whitfield and Richards, 1992). The lower threshold for larval development is

15°C, and 116 degree-days are required for complete development (Whitfield and Richards, 1992). Kemp and Bosch (2000) found that in diapausing individuals, a fluctuating thermal regime (14:27°C, 8:16 hours, mean 22°C) reduced the length of the prepupal and pupal stages after diapause compared with a constant temperature (22°C).

Females will frequently nest in cavities as narrow as 3–4 mm in diameter when offered a choice (O'Neill et al., 2010). However, offspring produced in larger diameter tunnels are larger (Stephen and Osgood, 1965a; O'Neill et al., 2010) and collect more pollen per foraging trip as adults than those reared in smaller diameter tunnels (Stephen and Osgood, 1965a). Studies have shown that cavities that are 6.4–7.2 mm in diameter and 100–150 mm deep are optimum for female and overall cell production (Richards, 1984a). Industry-standard nesting blocks do not always adhere to these specifications. For instance, drilled solid pine boards (120 × 15 × 7 cm) have tunnels that are 5 mm in diameter and 65 mm deep (Peterson et al., 1992). The most commonly used nesting medium is solid polystyrene blocks (M. Wendell, personal communication), and they come in two depths, 7.6 and 9.5 cm (Fig. 19.2, bottom). The second most commonly used nesting medium is grooved wooden laminates (Fig. 19.2, top). When offered a choice, the bees will typically prefer to nest in wood rather than polystyrene nesting materials and previously used nesting material rather than new (Pankiw and Siemens, 1974). Machines that push out the leaf-encased cocoons have been developed and are widely used (e.g., Pinmatic Inc., Pinawa, Manitoba; Fig. 19.3). Richards (1978) found a greater percentage of viable cocoons when the bees nested in grooved wood, Styrofoam, and polystyrene laminates compared to drilled boards, soda straws, particle board, aluminum, and corrugated paper. Tumblers are often used to remove debris-feeding insects, predators, and excess leaf pieces from stripped cocoons (Richards, 1984b).

Once the larval bees have reached the prepupal stage, there are physiological benefits to providing a prewintering period of a month or more at a cool temperature (e.g., 16°C) prior to wintering (Pitts-Singer and James, 2009). Leafcutting bees are best held in cold storage (5°C–8°C) over the winter to minimize mortality (Stephen and Osgood, 1965b). Kemp et al. (2004) found that survival of alfalfa leafcutting bees increased sharply with greater than 3 months of wintering and that weight was static during this period. Stripped cocoons or loose cells can be held in boxes, barrels, or trays. It is important to monitor the temperature in the center of the mass of cocoons, especially late in winter, because they can begin to warm prematurely. The heat given off by respiring immature bees can trigger those nearby to break diapause, and the container can warm considerably despite being in cold storage. This is of particular concern during shipping in the spring. If this situation occurs, the cocoons can be spread out in trays to allow them to cool back

FIGURE 19.2 Alfalfa leafcutting bees nesting in grooved wooden laminates (top) and polystyrene nests strapped back to back and stacked in a portable trailer in central California (bottom). For color version of this figure, the reader is referred to the online version of this book.

down. These cocoons would need to be sampled for developmental stage and mortality. Bees held in their natal nests are less prone to heating during cold storage; however, it is important to allow good air circulation around stacked nesting materials.

As pollination season approaches, an estimate must be made at least 3 weeks in advance as to when the bees will be needed in the field. Since the bees overwinter in the prepupal stage, the immature bees must molt into the pupal stage and then again into the adult stage. This process takes about 18−23 days for males and 21−25 days for females at 30°C. It is usually best to begin incubation about 30 days prior to the expected release date because adult bees can be cooled and held for up to 14 days at 15°C°C. However, they cannot be forced to develop any faster than they do at 30°C. The lower threshold for postdiapause development is 15.7°C (Richards and Whitfield, 1988), so at 30°C, 14.3°C days accumulate each day. It is useful to monitor temperatures and calculate degree-days, which can be compared to Table 19.1 to keep track of developmental events. A recent study showed that providing a 1 hour pulse of high temperature during the adult holding period can markedly increase the storage time period (Rinehart et al., 2011).

Usually, nests or lose cocoons that are beginning to emerge are taken to the field to complete the process. It becomes obvious when the bees are ready to be taken to the field because the sound of their chewing is audible. Cool

FIGURE 19.3 An alfalfa leafcutting bee cocoon extractor. This Dual Pinmatic Harvester is capable of harvesting 420 nests per minute (PINMATIC Inc., Pinawa, Manitoba). For color version of this figure, the reader is referred to the online version of this book.

TABLE 19.1 Alfalfa leafcutting incubation events by day of incubation (at 30°c) and degree-days.

Day	Degree-days	Comments
1	14.3	Cocoons to 30°C. Black-light traps in place.
7	100	Dichlorvos strips in; one strip per 38 m^3.
8	114	*Pteromalus* begin to emerge; bees begin to pupate.
10	143	Pink eye stage.
13	186	Remove dichlorvos strips and turn air out.
14	200	*Megachile relativa* (a native leafcutting bee) begin to emerge. Ignore these.
15	215	Incubator can be closed again. Temperature may be dropped to 10°C−15°C from now until day 22 to delay emergence.
18	257	Males begin to emerge.
21	300	Females begin to emerge.
22	315	Peak male emergence.
24	343	Peak female emergence.
28	400	Emergence is complete.

Source: Adapted from a "Calendar of Incubation" for alfalfa leafcutting bees by D. Murrell, Saskatchewan Agriculture and Food.

weather in the field can slow emergence, so to avoid this problem, some bee producers release emerging bees in the field in a method called a "bleed-off system" (Stephen, 1981). Incubating bees are allowed to emerge in the incubator, and they are then drawn to a light source, where they fall into a cold room and into containers with excelsior (wood shavings). The excelsior gives the bees something to cling to and prevents them from chewing on each other. This system has the advantage of generating a full population of flying bees immediately, but bees released this way have a greater tendency to abscond from the area.

Production of alfalfa leafcutting bees increased rapidly in the 1970s and 1980s. Alfalfa seed acreage also increased markedly, and from 1978 to 1980, leafcutting bees were being sold for over $185/10,000 bees (10,000 bees = 1 gallon in the alfalfa leafcutting bee industry). As acreage leveled off in the late 1980s and 1990s, prices fluctuated between around $50 to $100/gallon (Fig. 19.4). In 1999, several seed companies grew more alfalfa seed for markets in Argentina. Then, a financial crisis in that country left the seed companies with a surplus of alfalfa seed. This surplus lasted several years and resulted in a large portion of alfalfa seed coming out of production in the United States. The price of alfalfa leafcutting bees plummeted in 2000 to $25/gallon and reached a low in 2004 of $15/gallon. Finally, by the late 2000s, alfalfa seed acreage began to increase, and the price of leafcutting bees likewise has recovered. Since 2009, prices have been over $90/gallon. Alfalfa leafcutting bees are now also being used in some other crops, especially canola. The combination of commodity prices for alfalfa seed and canola can have a large influence on alfalfa leafcutting bee prices. When both commodities reach relatively high prices, as they have recently, more acres are planted; this in turn creates more demand for bees, and the price of bees rises.

In the United States, it is common to have fewer bees produced in alfalfa seed than were released there. Typical bee losses range from 50% to 80% (Pitts-Singer and James, 2005, 2008). In Canada, however, it is common to increase bees in alfalfa seed. Fourfold increases are known to have occurred, usually in smallscale, isolated locations. Usual increases are 1.5-fold to twofold in large-scale Canadian operations (D. Nahuliak, personal communication). The practice of releasing lower densities of bees in Canada, to encourage bee reproduction, seems to be the main explanation. Baird and Bitner (1991) recommend 50,000 to 100,000 bees per hectare (2–4 gallons/acre). Strickler and Freitas (1999) found growers stocking 78,000 to 130,000 bees/ha in Idaho. At these levels, pollination occurs rapidly, but floral resources decline rapidly as well (Strickler and Freitas, 1999). Bosch and Kemp (2005) found evidence that US alfalfa seed growers often stock too many bees in their fields and that the timing of release is late. When bee density is high compared to floral resources, nest establishment, pollination efficiency, and reproductive success are reduced (Pitts-Singer and Bosch, 2010). In Canada, 25,000–50,000 bees/ha are stocked, although fewer than that may be sufficient for good pollination (Parker et al., 1987). Other factors that help Canadians produce more bees include reduced pest and disease pressure, and a single generation of bees timed for a very intense bloom coinciding with very long days. Also, a lower percentage of cocoons produced in the United States contain live larvae, and survival of females is lower compared to Canadian bees (Pitts-Singer and James, 2005). Thus, a system has developed in which Canadian producers export their excess bees to the United States, where they are stocked at high density and replaced annually

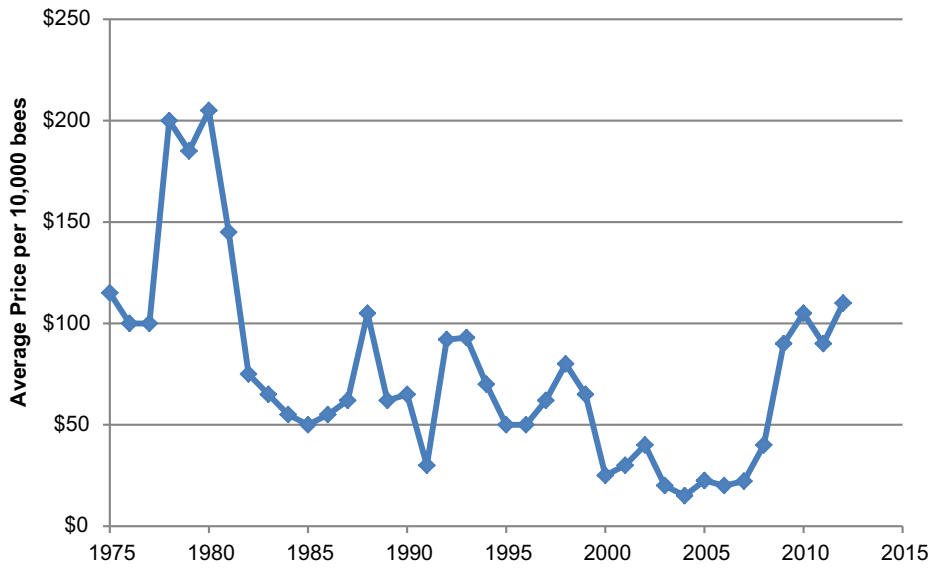

FIGURE 19.4 Prices for alfalfa leafcutting bees between 1975 and 2012 (Ron Bitner, International Pollination Systems). For color version of this figure, the reader is referred to the online version of this book.

(Parker et al., 1987). In 2011, Canada produced 355,126 gallons of leafcutting bees, down from 399,968 in 2006 (Census of Agriculture, 2011). At $90/gallon, the leafcutting bees produced in 2011 were worth over $31 million. Alberta produced 53%, Saskatchewan 35%, and Manitoba 11% of Canadian leafcutting bees.

Like any organism, the alfalfa leafcutting bee has a guild of natural enemies. Several of these can severely limit bee production if not managed. One of these is *Pteromalus venustus* Walker, a parasitoid in the family Pteromalidae. These small wasps (2.5 mm in length for females) parasitize the prepupal stage of the bee. The wasp has an ovipositor that can pierce through 1 mm to reach the larvae, and an average of 16 adult parasitoids (range = 7−26) will develop in a single bee larva (Richards, 1984a). From 1976 to 1983, parasitism levels averaged about 1% in western Canada, but they can reach as high as 54% (Whitfield and Richards, 1985). Cocoons are most vulnerable during incubation as the adults emerge around the 12th day of incubation. Females then can parasitize other leafcutting bees in the incubator. Fortunately, the wasps are attracted to ultraviolet light, where they can be drowned in a pan of water. In addition, dichlorvos strips introduced to the incubator are very effective in killing wasps (Hill et al., 1984). Strips are put into incubation chambers on day 7 (30°C incubation temperature) and removed on day 13, then fans are used to air out the chamber for 24−48 hours with no ill effects on the bees.

The fungal pathogen, chalkbrood, is caused by *Ascosphaera aggregata* Skow and is the most serious disease for leafcutting bee producers (James, 2011). Infected larvae grow to maturity but die before pupation. The dead larva turns black and is filled with billions of spores. As sibling leafcutting bees chew their way out of the nest, they must chew through these cadavers and become contaminated with the spores (Vandenberg et al., 1980). When these females begin to nest, they inadvertently leave spores in the nest provisions. In commercial pollination, cocoons are often removed from nesting substrates to reduce transmission, but this does not eliminate the disease (James, 2011). In Manitoba and Alberta, chalkbrood is prevalent, while Saskatchewan is relatively free of the disease (M. Wendell, personal communication). In order to keep Saskatchewan free of chalkbrood, it is common practice not to move leafcutting bees from Alberta or Manitoba into the province. In areas where chalkbrood is prevalent, there are methods to limit the disease buildup. Wooden boards can be redrilled and heated to 120°C to kill the spores (Kish and Stephen, 1991). For polystyrene nests, the fumigant paraformaldehyde is effective, but it is registered for use only in Canada (Goerzen, 1992) and Montana (EPA Reg. No. 4972-43, EPA SLN No. MT-12-0001). Dipping in chlorine bleach (sodium hypochlorite) is also effective on wooden boards and polystyrene nests (Mayer et al., 1988).

It is also common to find failed provision-filled cells or "pollen balls" in nests of the alfalfa leafcutting bee. These losses can vary from 4% to 42% and are more prevalent in the United States than in Canada (Pitts-Singer and James, 2008). These pollen balls appear to have multiple causes, such as hot weather or cool, wet weather (with the presence of fungus). In addition, high bee density can also increase the incidence of pollen balls (Pitts-Singer and Bosch, 2010).

Bee producers in Canada can have bees examined for pests, parasites, number alive per pound, machine damage, and sex ratio by the Canadian Leafcutter Bee Cocoon Testing Center (Brooks, Alberta). Batches of cocoons are imaged using X-rays, which readily show live and dead larvae, parasites, and diseased individuals (Stephen and Undurraga, 1976). A health certificate for each batch examined is generated that helps the industry monitor diseases and pests and allows buyers and sellers to negotiate a fair price for the bees.

The alfalfa leafcutting bee has a single generation in northern latitudes, but it can have a partial second or even third generation in more southern locations. For producers in Canada, the single generation may benefit bee health because chalkbrood and other parasites have less opportunity to reproduce in the field. In more southern latitudes, as in Idaho and California, a second generation is common; and in the San Joaquin Valley of California, it is not uncommon to have a third generation. With the long bloom of alfalfa seed (from late May to late July), two generations of bees can be beneficial for pollination. In fact, sometimes growers can sell second-generation bees when they have a crop that needs no further pollination to growers who have a late-blooming field. The trade-off in having multiple generations is that reproduction usually suffers from subsequent generations. The partial second generation is more likely to disperse due to waning bloom at that time of year, and the emerging bees may destroy diapausing bees as they exit the nest (Tepedino and Parker, 1986). Also, the second generation has a female-biased sex ratio, while the diapausing generation has a male-biased sex ratio (Tepedino and Parker, 1988). Hobbs and Richards (1976) selected a single-generation bee with success, but there has yet to be a consistently single-generation bee strain available to producers.

The alfalfa leafcutting bee was once thought of as strictly a pollinator for alfalfa and other legumes, such as sweet clover and birdsfoot trefoil seed production. However, over the years, the bee has been tried on many other crops, with some excellent results. For example, carrot seed in enclosures (Tepedino, 1997), blueberry (Stubbs et al., 1994), and canola (Soroka et al., 2001) have been successfully pollinated with alfalfa leafcutting bees. Recently, hybrid lettuce seed was successfully pollinated by alfalfa leafcutting bees (Gibson et al., 2007).

The alfalfa leafcutting bee is an excellent example of how a solitary bee can be employed to pollinate crops. Research and industry innovation have produced equipment and methods to make the bee a commercially sustainable pollinator. The industry has proven to be highly resilient to perturbations in the market, and agriculturalists continue to find new crops for it to pollinate. It is clear that the alfalfa leafcutting bee will remain an important managed pollinator in the future.

19.3 The alkali bee

N. melanderi (Fig. 19.5), the alkali bee, is a ground-nesting bee that is native to western North America and is in the family Halictidae. It can nest in dense aggregations in favorable habitats. It requires soils with 7%−8% clay content, a high water table, and a compacted surface (Mayer and Johansen, 2003) (Fig. 19.6). Mass producing this species can be as simple as planting alfalfa near an existing, natural bed and ensuring that the bees are protected from pesticide applications. Artificial beds can be constructed and "seeded" with cocoons or adult bees.

The alkali bee is just as efficient as the alfalfa leafcutting bee, tripping 78% of alfalfa flowers on single visit (Cane, 2002). Also, alkali bees are more likely to fly below the alfalfa canopy than other bees. Females dig holes up to 21 inches deep and construct individual cells branching from a main downward tunnel. Pollen is carried back to the nest on hairs on the hind legs. A female can construct 18−20 cells under ideal conditions, with half of her offspring being

FIGURE 19.5 The alkali bee, *Nomia melanderi*, on alfalfa blossoms. For color version of this figure, the reader is referred to the online version of this book. *Photograph by J. Cane.*

FIGURE 19.6 A managed alkali bee bed showing tumuli (piles of excavated soil). For color version of this figure, the reader is referred to the online version of this book. *Photograph by J. Cane.*

female (Stephen, 1960). During an optimal day, a female starts by laying an egg on a pollen provision collected the previous day, gathers and makes a new pollen provision, and then digs a new cavity for the next day (Stephen, 1959). A single generation develops annually in the Pacific Northwest, but multiple generations occur in California (Stephen, 1965). Stephen (1960) constructed artificial beds that produced population densities eight times higher than in the best naturally occurring beds after 2 years. Beds can become quite crowded with nests; for instance, one populous nesting bed carried a median density of 278 nests/m^2 (Cane, 2008a). Alkali bees will fly up to 3 miles away when floral resources are not nearby (Stephen, 2003).

During the 1960s, interest in alkali bees stimulated the installation of over 100,000 square feet of artificial bee beds in one water district in central California (Wichelns et al., 1992). Statistical analysis showed that the alkali bees had a positive impact on alfalfa seed yield during that time (Wichelns et al., 1992); however, the beds declined suddenly in the 1970s, most likely due to changing pesticide use in the area.

Because alkali bees are soil-nesting bees, there are some challenges involved in establishing new beds and in moving the bees. Blocks of soil containing nests can be cut from existing beds and transplanted into new ones in the spring (Johansen et al., 1982). A steel coring tool, 25−30 cm in diameter, is driven into the soil using a tractor or human power, or a large number of cores can be cut with a circular saw blade mounted on a back hoe. The cores can then be transported and buried into a new bed. Newly emerged bees can also be captured, anesthetized with CO_2, and released at a new site in the evening (Johansen et al., 1982).

The number of managed alkali bee beds peaked in the late 1950s and early 1960s then began to decline. Mayer and Johansen (2003) attribute the decline to the rise in interest in, and competition from, the alfalfa leafcutting bee. They showed that competition from other bees in the field reduces alkali bee foraging. In addition, the success of the alkali bee beds can be set back severely by untimely rain storms, which occurred several years in a row during the 1960s. Stephen (2003) agreed that untimely spring rains led to the decline of managed alkali bees, and added the economics of bed construction and maintenance, predators and disease, pesticides, and the fouling of continuously reused nesting media.

Alkali bees continue to be used as pollinators in the Touchet Valley of Washington state, where there are at least 56 nesting sites (Cane, 2008a) supporting 17 million females. The most populous of these sites grew to a population of 5.3 million females. The continued success of these nesting beds in this area can be attributed to careful management of nesting beds by growers and because the primary crop in the Touchet Valley is alfalfa seed. The other rotational crops grown in the valley (wheat, field peas, and chickpeas) are not sprayed with insecticides. Growers are conscientious about any insecticide application on the alfalfa seed, applying them before or after bloom (Cane, 2008a). Yields in fields pollinated by alkali bees are comparable to those pollinated by alfalfa leafcutting bees. As the pollination service provided by the alkali bees has been sufficient there, growers do not bring in alfalfa leafcutting bees or honey bees, so competition for floral resources is minimized. Growers who utilize these bees find that after the initial investment in bee beds, the management requirements are minimal.

19.4 The blue orchard bee

The blue orchard bee or orchard mason bee, *O. lignaria* (Fig. 19.7), is native to North America and occurs throughout much of the United States. Two subspecies are recognized: *O. lignaria lignaria* Say occurs east of the 100th meridian, while *O. lignaria propinqua* Cresson occurs to the west (Bosch and Kemp, 2001). Females are usually slightly larger than a honey bee and dark metallic blue to blue-green in color. These bees nest in preexisting cavities in dead wood (abandoned nests of wood-boring beetles and hollow plant stems), but sometimes in paper or mud nests of wasps, ground nests of bees, or *Xylocopa* (carpenter bee) nests (Cane et al., 2007). Blue orchard bees overwinter as adults inside the cocoon and emerge early in the spring, usually when daytime high temperatures begin to exceed 20°C. They visit a wide variety of plant species in 34 plant families (Bosch and Kemp, 2001). The blue orchard bee is strictly univoltine.

In 1970, Philip Torchio made a survey of bees present in apple and pear orchards in Utah and surrounding states. He found that *O. lignaria* was omnipresent in these orchards and would be a good candidate for development as a commercial pollinator (Torchio, 2003). Field trials showed that blue orchard bees would successfully nest and pollinate in almonds (Torchio, 1979, 1981a,b, 1982a), apples (Torchio, 1982b,c, 1984a,b, 1985), prunes (Torchio, 1976), and cherries (Bosch and Kemp, 1999; Bosch et al., 2006). Fivefold increases in female populations have been documented in a pear−apple orchard (Torchio, 1985) and in cherries (Bosch and Kemp, 1999). In Oregon and Washington, cherry and apple growers are using blue orchard bees successfully. Other crops, such as meadowfoam (Jahns and Jolliff, 1991), pear, plum, prune, peach, nectarine, strawberry, currant, gooseberry, blackberry, rape, and canola, are also good candidates for commercial pollination by blue orchard bees (Torchio, 1976; Bosch and Kemp, 2001).

Mass producing the blue orchard bee involves providing cold storage for the wintering cocoons followed by release in the orchard. Since this species overwinters as an adult, the incubation period is much shorter than with the alfalfa leafcutting bee. Cavities must be provided in wood or paper tubes, and a source of moist soil must be nearby. For good reproduction, nectar and pollen must be present for 3–4 weeks. Offspring cocoons need to be monitored closely in order to begin the wintering period not long after they have reached the adult stage.

Blue orchard bees will accept artificial tunnels (Levin, 1957), but they prefer to nest in holes 6–7 mm in diameter (Torchio, 2003). In cavities less than 10 cm in length or narrower than 6 mm, nesting females will produce a greater proportion of male offspring. Drilled wooden blocks are the most attractive nests, and nesting is improved when holes are separated by more than 12 mm (Torchio, 2003). Visual, as well as olfactory cues, are important when selecting a cavity, and the odors from old female cocoons are attractive (Pitts-Singer, 2007). A typical nest will contain 2–3 females and 4–6 male cells (Fig. 19.8). Female cells are constructed first at the back of the nest and are about 1.7 times as large as a male cell. When foraging for nest provisions, blue orchard bees will collect both pollen and nectar. Pollen is packed onto the abundant bristles of the scopa on the underside of the abdomen just as does the alfalfa leafcutting bee. When the female returns to her nest, she enters head first and disgorges nectar onto the lump of pollen (Torchio, 1989). She then backs out, turns around, and backs into the cavity so that she can unload the pollen using her hind legs. Once the pollen provision is large enough, the female will make a final foraging trip for nectar and deposit a small pool of nectar on top of the pollen mass. She then turns around and oviposits on the pollen provision. Adjacent cells are separated by mud partitions, and mud is also used to form a plug at the end of the completed nests (Torchio, 1989).

The egg stage lasts about 7 days at 22°C (Bosch and Kemp, 2000). The first instar consumes embryonic fluids inside the egg and molts into the second instar as it encloses from the egg chorion (Torchio, 1989). The second instar emerges

FIGURE 19.7 The blue orchard bee, *Osmia lignaria*, on an almond blossom. For color version of this figure, the reader is referred to the online version of this book.

FIGURE 19.8 A blue orchard bee nest in a split reed with pollen–nectar provisions, mud partitions, and eggs. Larger provisions will become females (five in this nest). For color version of this figure, the reader is referred to the online version of this book.

from the egg and proceeds to feed on the pollen−nectar provision. The larva progresses through five instars in about 21 days at 22°C (Bosch and Kemp, 2000). Cocooning takes about 5 days, and then a prolonged prepupal stage of about 29 days occurs. After pupation, the immature bee spends about 32 days in the pupal stage before molting to the adult stage. Development from egg to adult takes approximately 87 days at 22°C (Bosch and Kemp, 2000). Developmental rates are highly dependent on temperature. For example, at 18°C, egg-to-adult development takes more than 123 days, whereas at 29°C it takes only about 63 days (Bosch and Kemp, 2000). A fluctuating temperature regime, averaging 22°C, gave the highest survival, and development was completed in 70 days. Blue orchard bee prepupae take longer to develop at temperatures above or below 26°C (Kemp and Bosch, 2005). As temperatures begin to drop in the fall, the adult enters diapause, still inside the cocoon. Overwintering as an adult is a derived trait and makes these bees better able to take advantage of early spring blooms (Bosch et al., 2008).

Bees from warmer climates (e.g., at low elevation in California) typically have a longer prepupal stage where they enter a summer diapause, or estivation, compared to bees from cooler climates (like Utah) (Bosch et al., 2000). Cocoons can be wintered artificially at 3°C−5°C successfully (Bosch and Kemp, 2003), and at least 3 months of wintering are required for best survival rates (Kemp et al., 2004). Although blue orchard bee adults reduce their metabolic rate as soon as they become adults, they lose considerable weight during prewintering (Kemp et al., 2004; Bosch et al., 2010; Sgolastra et al., 2011). Sgolastra et al. (2010) found that diapause can be divided into two phases. The first phase lasts until about 100 days of wintering have elapsed and is an intense diapause (when incubated, emergence time is prolonged). In the second phase, after 100 days, increasing the wintering temperature can rapidly decrease the diapause intensity and promote emergence from the cocoon. Even with continued winter temperatures, diapause intensity gradually diminishes in this second phase.

In the wild, warming spring temperatures will break diapause, and the bees will chew their way out of their cocoons and through the mud partitions, with males preceding females. In artificial management, cocoons or nests are warmed up, usually a week or two prior to bloom, to promote emergence. After 150 days of wintering at 4°C, average male emergence will occur in under 2 days at 20°C, and under 8 days for females (Bosch and Kemp, 2000). Males will patrol the area, feeding on nectar and sleeping in crevices until the females emerge. Females apparently emit a sex pheromone (virgin females give off a distinctive odor), which attracts the males. Mating usually occurs close to the natal nest, but sometimes on nearby vegetation.

Females take 2−3 days to feed on nectar and pollen and search for suitable nesting sites. Once they have selected a suitable cavity, they usually first collect mud and make a partition at the back of the cavity, and then proceed to collect a pollen−nectar provision. Females can be observed spiraling around the tunnel as they exit their holes, apparently marking it with a secretion from the Dufour's gland (Guédot et al., 2006; Pitts-Singer et al., 2012). This marking ensures that they have the correct hole when they return. Changing or cleaning the cavity entrance while the bee is foraging results in a disruption of the unloading behavior; this indicates that odor cues are important for the bee in identifying its nest (Guédot et al., 2006).

One-half to one cell is usually completed in one day during normal field conditions, but two per day are possible (Torchio, 1989). A foraging trip typically takes 5−10 minutes, and a female will visit about 75 flowers in an average trip (Bosch and Kemp, 2001). A single provision may require 20−30 foraging trips. When the series of cells is complete, the female constructs a thick (8−9 mm) plug at the opening of the cell to seal the nest and prevent predators or scavengers from entering. A female bee can live up to 6 weeks, but 3−4 weeks is typical. Under ideal conditions, a female can produce up to 32 eggs, 10 of which would be daughters, but it is more typical for a female to construct about two complete nests with about 4−6 daughters.

Management of these bees is relatively simple, but two life stage events require close monitoring for success when artificial storage conditions are used. These are (1) the onset of wintering in the autumn, and (2) the timing of emergence in the spring (Bosch et al., 2008). If the bees experience a lengthy prewintering period (>3 weeks), they can become weakened, so knowing when the bees reach adulthood is critical for timing winter storage. A sample of nests can be X-rayed to monitor development, or a sample of males can be sacrificed by opening the cells to determine stage of development. Emergence timing can vary greatly with the length and temperature of wintering (Bosch and Kemp, 2003; Bosch et al., 2008). At least 150 days of wintering are required for prompt emergence of females (Bosch and Kemp, 2003). To estimate the amount of time until bee emergence, a sample of bees can be incubated 2−3 weeks prior to the expected bloom (Bosch and Kemp, 2001).

Commercial pollination with blue orchard bees requires large numbers of bees. Managers of the species have three options for increasing their population: (1) Trap wild bees or purchase trapped bees, (2) increase populations in the orchards where the crop is pollinated, and (3) propagate bees away from orchard sites. Acquiring enough blue orchard bees through wild trapping cannot meet customer demands, is often unpredictable, and can have negative consequences for wild bee populations (Roulston and Goodell, 2011). Propagation away from orchards often called "bee ranching,"

offers the advantage of longer bloom times, depending on the flowers available, and separation from agricultural chemicals. Blooming plants are known to be visited by blue orchards bees can be planted, and they can even be enclosed in a greenhouse or screenhouse (Fig. 19.9). A screenhouse enclosure ensures that the bees do not disperse and eliminates honey bees as competitors for the crop. The annual wildflower, *Phacelia tanacetifolia* Bentham (Fig. 19.9, inset), makes an excellent forage crop for the bees. *Collinsia heterophylla* Buist *ex* Graham and *Nemophila maculata* Bentham *ex* Lindley are also good pollen and nectar producers and are highly attractive to the bees. Increases of up to 4.5-fold have been achieved in a screenhouse with wildflowers (S. Peterson, unpublished data). With a single generation per year, propagating bees on wildflowers means forgoing a pollination fee so that a larger population is available for pollination in subsequent years.

In California, almonds have steadily increased in acreage. By 2011, there were approximately 338,000 ha of almonds, with a production value of $3.5 billion (USDA National Agricultural Statistics Service, 2011), and California is one of the few places in the world where the climate is favorable for production. Almonds are especially dependent on bees for successful pollination (McGregor, 1976), but they are among the earliest crops to bloom, typically starting around February 15 during California's rainy season. Typically, 4.9 colonies of honey bees are recommended for almond pollination per hectare (Thorp, 1996). This amounts to 1.5 million colonies of honey bees or about two-thirds of the entire US supply. The cost of renting honey bees in almonds more than doubled from 2004 to 2006 (Sumner and Boriss, 2006) due to rising demand from increasing almond acreage and reductions in honey bee supply, largely caused by honey bee pests (including parasitic mites, fungal and bacterial diseases, and viruses) and colony collapse disorder (CCD). Since that time, prices have remained at these levels (in a range of $120 to $150/colony). Rental prices are not expected to drop back to previous levels because the bearing acreage of almonds continues to increase, but the number of honey bee colonies is not increasing. The National Research Council (2007) documented the decline in honey bee colonies in the United States and determined that pests, pathogens, and environmental stressors (e.g., pesticides, transportation, and poor nutrition) may be contributing to the decline.

While it had been known since 1975 that blue orchard bees can pollinate almonds efficiently, the cost to provide blue orchards bees was not competitive with costs for honey bees. However, with honey bee rental fees currently at or near $740/ha, interest in alternative pollinators has increased greatly. Blue orchard bees have shown to be highly effective at pollinating almonds (Torchio, 1979), requiring far fewer bees (Bosch and Kemp, 2002) and possessing the ability to forage under worse conditions than the honey bee (Bosch and Kemp, 1999). In addition, a recent study showed that a combination of blue orchard bees and honey bees produces a greater proportion of fruit set than either species of bees alone (Brittain et al., 2013). The blue orchard bee is clearly an attractive candidate as an alternative or a supplement to honey bee pollination in almonds.

At this early stage of using blue orchard bees in almonds, progress has been hampered by the limited supply of bees and low reproduction levels in large-scale releases, but this may soon be alleviated with the emergence of a growing number of suppliers committed to improved processes and products, as well as reduced costs (see http://www.orchard-bee.org). For blue orchard bee pollination to expand further in almonds, additional enabling technology is needed to enhance in-orchard female offspring production, as has been demonstrated in apples, cherries, and pears. A reasonable assumption is that each established female can produce two female offspring in an almond orchard (Bosch and Kemp, 2002). This means that at least one-half of the females released must establish nesting for replacement of the parent population. AgPollen LLC, in collaboration with the US Department of Agriculture's Agricultural Research Service

FIGURE 19.9 A 5-acre screenhouse dedicated to propagating blue orchard bees in central California. Inset: *Phacelia tanacetifolia* blossom and a blue orchard bee female. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

(USDA-ARS), is working on attractants for nesting structures to improve establishment (S. Peterson et al., unpublished data). Methods for releasing bees and providing nesting substrates are improving, but they have not been standardized yet for large-scale applications.

Almonds can be a challenging crop to pollinate even for the honey bee. Almonds bloom for 3–4 weeks at best, with the middle 2 weeks at full bloom, so there is little time for the bees to nest and produce offspring with this resource alone. In addition, weeds and vegetation are typically treated with herbicides or mowed, so there is usually little else in bloom near the orchards. While honey bees can be fed syrup and protein patties before almond bloom, blue orchard bees must have flowers available upon release. Blue orchard bees may abscond if released too early without enough bloom. When blooming is finished, the bees are usually still faced with a lack of other food sources. Efforts are underway to find wildflowers that can be planted in or near almond orchards that can provide supplementary bloom before and after almond bloom (http://www.projectapism.org; http://www.xerces.org; S. Peterson, unpublished data) to improve bee health and reproduction.

Rain can stimulate brown rot blossom blight and other fungal diseases, so preventative fungicides are commonly applied while bees are in the orchard. Often, foliar fertilizers are added with these applications. More rain falls in the northern almond-growing regions, so this problem is more common in the northern and central regions of California. While most fungicides are not acutely toxic to bees, there may be sublethal effects on nesting females and developing larvae. Commercial fungicide applications have been observed to cause disruptions in foraging and nesting behavior in blue orchard bees (Ladurner et al., 2008) and, when tested in cages, caused significant disorientation after the application (Artz et al., unpublished data). In addition, trace amounts of insecticides may find their way into bee nests. A sample of California almond pollen collected by blue orchard bees in 2011 was analyzed for chemical residues, and two fungicides, a pyrethroid and three neonicotinoid insecticides, were detected (S. Peterson, unpublished data). None of these pesticides had been sprayed in that orchard. Pesticide drift or other types of movement (e.g., windborne dust particles) is clearly a possibility that can impact bee pollination and reproduction in an orchard. Because mud is used as a partition between cells, pesticides in the soil are also of concern. More research is needed on the sublethal effects of pesticides (singly and in combinations) and foliar fertilizers on bees. Pollination providers need to communicate with growers to ensure that fungicides are applied at night and to make sure that the spray tank has been properly cleaned so that no insecticide residues are present.

Blue orchard bees normally fly a month later than almonds bloom. However, populations can be manipulated with warmer or fluctuating temperatures during the summer to complete development as soon as possible, which allows an earlier wintering starting date (Bosch et al., 2000). A battery-powered incubation box was developed that can advance emergence despite cool weather conditions in the orchard (Pitts-Singer et al., 2008). Since the bees require some incubation to emerge, time can be lost by emerging them in the orchard, so releasing emerged bees (like the "bleed-off" in alfalfa leafcutting bees) has been tried successfully in almonds (M. Allan and S. Peterson, unpublished data). Additionally, offering many smaller nest sites improves establishment compared to fewer sites per unit of area (Torchio, 1984b; Artz et al., 2013). Recent work showed that nest box density and the number of cavities within nest boxes influenced blue orchard bee retention in almonds (Artz et al., 2013). Females completed more nests in high-density, low-cavity nest boxes compared to low-density, high-cavity nest boxes. Nut yield was also significantly higher in orchard areas with high-density nest boxes. A strategy of providing both honey bees and blue orchard bees, each at roughly one-half the usual stocking density, allows the artificially fed honey bees to pollinate the very first blossoms. The blue orchard bees can be introduced once enough bloom is available to support the population (e.g., 5%–10% bloom). If growers plant wildflowers that bloom ahead of almonds, honey bee colonies would likely be stronger and blue orchard bees could be released earlier.

The recommended stocking density for blue orchard bees in almonds is 740 nesting females per hectare (Bosch and Kemp, 2001). To achieve this rate requires estimating losses from mortality and lack of establishment, which at present can be 50% or more in large-scale applications. Therefore, it is usually best to stock at least twice the recommended rate. If honey bees will also be present, then a reduced rate can be justified.

As is the case with the other pollinator species discussed in this chapter, the blue orchard bee is subject to a guild of parasites, predators, and diseases. Lessons learned with the alfalfa leafcutting bee are relevant in keeping managed blue orchard bees healthy. The fungal disease chalkbrood (*Ascosphaera torchioi* Youssef and McManus) has a similar life cycle in the blue orchard bee compared to chalkbrood of alfalfa leafcutting bees (Bosch and Kemp, 2001). Chalkbrood rates in blue orchard bees over a 15-year period in Utah ranged from 0.4% to 2% (Torchio, 1992). Allowing bees to emerge from natal nests could allow chalkbrood to increase in a population just as in alfalfa leafcutting bees. Loose cell management allows for the visual inspection and removal of infected cadavers. So far, this disease has remained at low levels in managed populations.

Several hymenopteran parasitoids and cleptoparasitoids attack the blue orchard bee. *Monodontomerus obscurus* Westwood and *M. montivagus* Ashmead (Hymenoptera: Torymidae) are commonly associated with blue orchard bees (Bosch and Kemp, 2001). Their ovipositor is about 1 mm in length, so when using paper tubes, walls of at least 1 mm thickness will prevent oviposition (Bosch and Kemp, 2001). Adults emerge from blue orchard bee nests during the spring and summer, and adults can be attracted to a black-light water trap and drowned. A study on the European species *M. aeneus* (Fonscolombe) showed that the odor cues of *Osmia* frass and cocoon are utilized by these parasitoids to find their hosts (Filella et al., 2011). Perhaps such cues can be used to trap and kill these pests.

The sapygid wasp, *Sapyga* spp. (Hymenoptera: Sapygidae), is a cleptoparasite and is commonly associated with blue orchard bees. Cleptoparasites lay their eggs in open bee nests, and the larvae consume the pollen provision and kill the developing blue orchard bee larvae. These wasps have a single generation per year and emerge shortly after or with the female blue orchard bees. To control this pest, nests must be visually inspected and the *Sapyga* cocoons manually destroyed. While *Sapyga* cocoons look somewhat different from blue orchard bee cocoons, they still can be overlooked when one is working with large numbers of cocoons. Careful inspections are necessary to eliminate this species from a population.

Stelis montana Cresson (Hymenoptera: Megachilidae) is a cuckoo bee and, like the sapygid wasp, is a cleptoparasite. Cuckoo bees lack the pollen-collecting scopae found on other Megachilid females and can parasitize several *Osmia* species. *S. montana* has a single generation per year and emerges slightly later than blue orchard bee females. It looks similar to the blue orchard bee, but is smaller in size and has a proportionately smaller head. *S. montana* cocoons are smaller and lighter colored, and they have long, coiled frass that distinguishes them from blue orchard bee cocoons. Visual inspection of cocoons is the best way to remove this species.

The hairy fingered mite, *Chaetodactylus krombeini* Baker (Acari: Chaetodactylidae), is a cleptoparasite with multiple generations per year. They attach themselves to emerging bees and are transferred to new nests in this manner. Infested cocoons can be removed individually, or cocoons can be washed in a bleach solution to remove them. Hairy fingered mites are most commonly a problem in cool, humid environments such as in Oregon and Washington. A similar species of mite on *Osmia cornifrons* (Radoszkowski) was controlled using warm temperatures (30°C−40°C) in Japan (Yamada, 1990). A protocol has been developed to control hairy fingered mites on blue orchard bees (E. Sugden, personal communication). For best results, nests are brought in from the field soon after completion, or a little before, and the temperature is increased to 27°C−32°C and humidity is reduced to 30%−40% for a few days. Nighttime temperatures are then allowed to cycle to ambient until the bees pupate. Tumbling cocoons with coarse sand can also be effective (J. Watts, personal communication). White et al. (2009) showed that the fumigants formic acid and wintergreen oil have the potential to control *C. krombeini* on *Osmia cornifrons* (Radoszkowski). Although we have highlighted the main blue orchard bee pests, several others are found associated with blue orchard bees; for complete descriptions with photographs, see Bosch and Kemp (2001).

Concerns over declining honey bee populations have stimulated interest in the blue orchard bee, and it is rapidly becoming a commercial pollinator of important fruit and nut crops. We expect that managed populations of this bee will grow greatly in the coming years. For this species to gain more widespread use, further research is needed on methods to minimize prenesting dispersal, the effects of sublethal doses of pesticides, the diversification of floral resources in orchards, and methods to advance large-scale management processes.

19.5 Other solitary bees of interest for pollination

A number of other bees besides those discussed in this chapter are used in pollination or have potential for mass rearing toward commercial pollination. For example, studies have shown that a North American species, *Megachile pugnata* Say, is an excellent pollinator of sunflowers (Parker and Frohlich, 1983). *Osmia sanrafaelae* Parker is a good pollinator of alfalfa seed and will trip 44% of alfalfa flowers when allowed single visits (Cane, 2002). *Osmia bruneri* Cockerell is native to western North America, nests in preexisting holes, and will use artificial domiciles (Frohlich, 1983). This species has the potential to be a commercial legume pollinator (Cane, 2008b). *Osmia ribifloris* Cockerell is found throughout the western United States and has the potential to be a commercial pollinator of blueberries (Torchio, 1990; Stubbs et al., 1994). *Osmia aglaia* Sandhouse is an excellent candidate in the western United States for commercial pollination of raspberry and blackberry crops (Cane, 2005a,b).

Outside of North America, other solitary bees are being used for commercial pollination as well. *Osmia cornuta* (Latreille) is a European species, like *O. lignaria*, that has been shown to be commercially manageable for almond and pear pollination in southern Europe (Bosch, 1994a,b; Krunic et al., 1991; Maccagnani et al., 2007). A highly effective pollinator of almond flowers, the body of *O. cornuta* contacted the stigma 98.7% of the time compared to 67.3% of the

time with the honey bee (Bosch and Blas, 1994). Single-visitation fruit set was also higher with *O. cornuta* compared to honey bees.

In Japan, *O. cornifrons* has been commercially managed for apple pollination for about 40 years (Maeta and Kitamura, 1974), and it is a more effective apple pollinator than the honey bee (Maeta and Kitamura, 1981). This species, known as the mame-ko bee or Japanese horn faced bee, was introduced to the United States in 1977 by the USDA (Parker et al., 1987). This species can double or triple its population each year (Adams, 2004). Thus far, the Japanese horn faced bee is managed in the eastern and north-central United States, but it has not been made available on a large commercial scale.

Osmia rufa (L.), the red mason bee, is another European species that can be used to pollinate seed crops in greenhouses (van der Steen and de Ruijter, 1991), and it has been shown to gather *Brassica* pollen and increase its population on the crop in Poland (Teper and Biliński, 2009). Also, the red mason bee is an effective pollinator of strawberry (Wilkaniec and Radajewska, 1997) and is currently being managed to pollinate cherries, apples, and pears in Great Britain (C. Whittles, personal communication).

19.6 Concluding remarks

The honey bee is unmatched in its abilities to pollinate a wide variety of crops, adapt to a wide variety of climates, and provide services year-round in warm climates. However, recent history has shown that reliance on this single species for crop pollination has its risks. For the past 30 years, the number of honey bee colonies has been shrinking in the United States due to a suite of factors, including parasites, diseases, poor nutrition, pesticide contamination, and CCD (National Research Council, 2007; Mullin et al., 2010; Ratnieks and Carreck, 2010), while the acreage of bee-dependent crops has been growing. Honey bees will always be an important part of agriculture, but it is becoming increasingly apparent that there are benefits in managing some other bee species to share in the job of crop pollination. The relatively recent adoption of the alfalfa leafcutting bee and alkali bee as commercial pollinators for alfalfa seed, and the burgeoning commercial use of the blue orchard bee in almonds, apples, and cherries, has demonstrated that solitary bees can be managed in an agricultural setting and provide sustainable pollination services.

Species that make their nests above ground, in preexisting cavities, make particularly good candidates for use in agricultural settings. However, soil-nesting species are also possible to manage. Solitary bees have shorter activity periods compared to honey bees, so adult bees do not need to be managed year-round. With blue orchard bees and alfalfa leafcutting bees, the population is held in storage, preferably under controlled conditions, for a large part of the year. With the alkali bee, developing bees are underground most of the year. These bees are highly efficient pollinators during their adult stage, and fewer individuals are needed to pollinate a given area compared to honey bees. With few management steps, short activity periods, and fewer bees needed, these bees have minimal negative impacts on the environment. As our scientific and practical knowledge of these species expands, solitary bees will become increasingly important commercial pollinators.

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Chapter 20

Production of bumblebees (Hymenoptera: Apidae) for pollination and research

Genevieve Rowe[1], Mallory A. Hagadorn[2], Thuy-Tien T. Lindsay[3], Rosemary Malfi[4], Neal M. Williams[5] and James P. Strange[6]

[1]*Wildlife Preservation Canada, Guelph, ON, Canada,* [2]*Department of Biology, Utah State University, Logan, UT, United States,* [3]*USDA-ARS Pollinating Insects Biology, Management and Systematics Research Unit, Logan, UT, United States,* [4]*Department of Biology, University of Massachusetts, Amherst, MA, United States,* [5]*Department of Entomology and Nematology, University of California, Davis, CA, United States,* [6]*Department of Entomology, The Ohio State University, Columbus, OH, United States*

20.1 An introduction to rearing bumblebees

Bombus (Apidae) is a genus of approximately 265 species of mostly eusocial bumblebees that occur throughout most temperate regions of the world. Domestication of bumblebees has long been discussed (Sladen, 1912; Plath, 1923; Frison, 1927); although, serious efforts to rear bumblebee colonies for pollination began in earnest in the 1980s (Van Heemert et al., 1990; de Ruijter, 1996; Velthuis and van Doorn, 2006). The realization that bumblebees are efficient pollinators of greenhouse crops, especially tomatoes (Van den Eijnde et al., 1991), and acclimated well to confined production systems spurred the development of year-round rearing systems to meet the pollination needs of this industry. As the commercial bumblebee industry has grown, so has the list of crops that use colonies for pollination services. Both published and unpublished reports indicate their use for pollinating avocado, blueberries, cranberries, canola, clover, cucumber, eggplant, pepper, pumpkin, raspberry, and strawberry, among others (Velthuis and van Doorn, 2006, and references therein).

Although commercial pollination is the primary driver for most mass rearing of bumblebees, several other factors have sparked a growing interest in rearing some species for research and conservation efforts (Plowright and Jay, 1966; Evans et al., 2007). Researchers have long used commercially available bumblebees for both field studies and laboratory experiments; however, the applicability of these studies as representative of bumblebee biology, in general, is always a question. This is because only a few species are widely available for use, limiting inference of studies that use commercial colonies to especially hardy and adaptable species. Commercially reared lines of these species may also differ from their wild conspecifics owing to generations of captive breeding. Interest in studying bumblebees in their natural habitat, as well as understanding differences in species behavior, life histories, and physiology, has led many researchers to rear bumblebees in their own labs, with varying degrees of success (Plowright and Jay, 1966; Evans et al., 2007; Kearns and Thomson, 2001; Salvarrey et al., 2013; Strange, 2015; Ptáček et al., 2015; Carnell et al., 2020b). In addition to the desire for locally sourced research colonies, there is growing interest in rearing bumblebees of rare and declining species both to understand the causes of declines and in hopes that wild populations can be augmented and/or reintroduced using captive-reared colonies (Smith et al., 2020). Over 30% of species in North America show some level of range contraction or decline in abundance (Fig. 20.1) and ex situ rearing has been proposed for species facing demographic challenges (Smith et al., 2020).

Bumblebee species have adapted to diverse climates and flora worldwide. Despite this global diversity, only a fraction of species has been developed for commercial pollination (Velthuis and van Doorn, 2006; Evans, 2017). In much of the world, *Bombus terrestris* (Linnaeus) is the commercial pollinator of choice due to well-established methodology for rearing developed decades ago by companies like Biobest, Koppert, and others (Velthuis and van Doorn, 2006;

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Dafni et al., 2010). In North America, the primary commercial bumblebee is *Bombus impatiens* Cresson, which is native to much of the eastern part of the continent (Williams et al., 2014). There, governments have restricted the use of non-native bumblebees like *B. terrestris* (Winter et al., 2006); nonetheless, *B. impatiens* is widely used in Canada, Mexico, and the United States outside of its native range. This practice has led to escapes from greenhouses and subsequent invasions (Ratti and Colla, 2010; Looney et al., 2019). Likewise, where *B. terrestris* has been transported for pollination, it has frequently escaped confinement and established in surrounding landscapes (Inoue et al., 2008; Geslin and Morales, 2015), sometimes with disastrous ecological impacts (Goulson, 2010a; Evans, 2017). Additionally, commercial bumblebees have been implicated in pathogen movement, as either vectors of disease to new locations (Goka et al., 2000) or amplifiers of disease around rearing facilities (Colla et al., 2006; Otterstatter and Thomson, 2008; Murray et al., 2013). In response to these issues, conservationists have begun advocating for locally sourced and produced bumblebee colonies (Strange, 2015; Evans, 2017).

20.2 Bumblebee lifecycle

Successful captive rearing of bumblebees requires some knowledge of the natural lifecycle (Fig. 20.2) that needs to be replicated in captivity. With the exception of several socially parasitic species (subgenus: *Psythirus*), bumblebee species are social insects with colonies that consist of a single queen, nonreproductive female workers, and reproductive males (Alford, 1975; Goulson, 2010a); for commercially developed species, worker populations can exceed 1000 individuals per colony (Table 20.1). Reproductive females prior to eclosion of their own offspring are termed gynes and may be

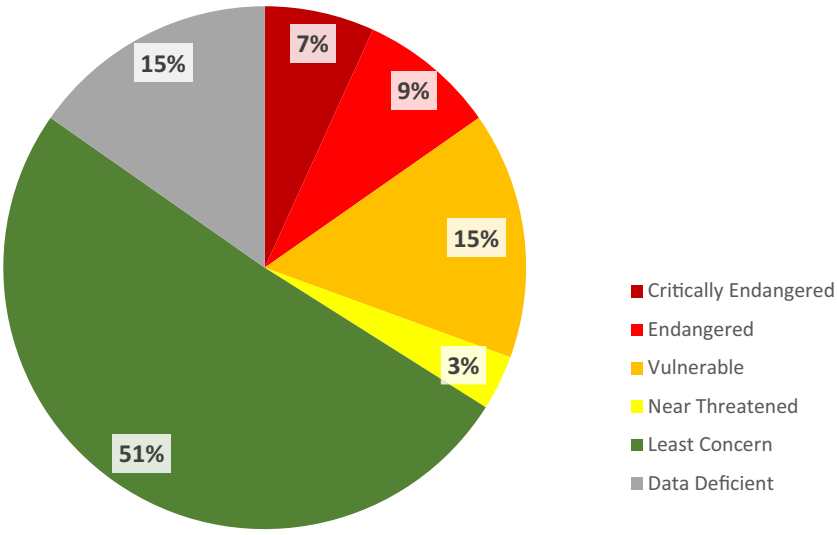

FIGURE 20.1 Proportions of Nearctic bumblebee species by conservation status as classified by the International Union for the Conservation of Nature Red List Status for Nearctic Bumblebee Species. Species status was based on conservation status from IUCN Red List of Species as of December 15, 2020 (IUCN 2020).

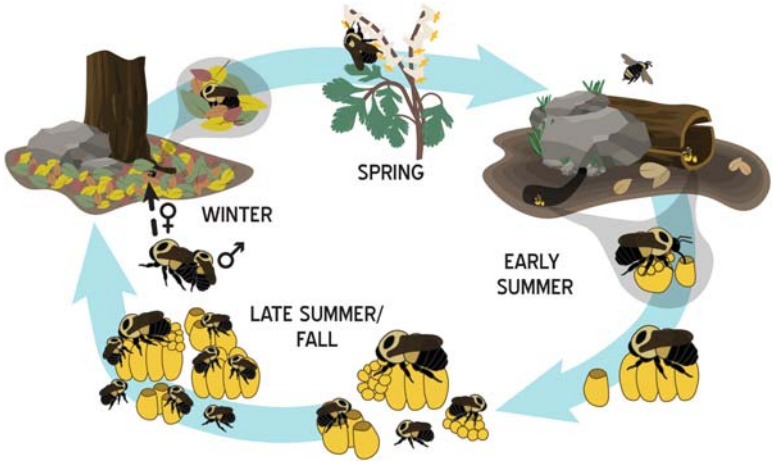

FIGURE 20.2 Diagram of the bumblebee colony life cycle. Timing of each successive stage varies depending on the species of bumblebee. *Artwork by Jeremy Hemberger.*

TABLE 20.1 Success rate (%) and relative colony size of selected western North American *Bombus* species raised at the USDA-ARS Pollinating Insect Research Unit from 2010–2019.

Bombus species	Success rate (%)	Relative colony size
appositus Cresson	53.8	Small
centralis Cresson	26.1	Medium
griseocollis (Degeer)	44.7	Small
huntii Greene	20.8	Very large
melanopygus Nylander	1.8	Small (medium)
mixtus Cresson	1.8	Small (medium)
nevadensis Cresson	5.6	Small
occidentalis Greene	44.4	Large (very large)
vancouverensis Cresson	32.5	Medium
vandykei (Frison)	29.5	Medium
vosnesenskii Radoszkowski	33.7	Very large

Notes: Success rate reflects the number of wild-caught gynes that produced a female offspring in the lab. Relative colony size is the average colony size of the total output of males, workers, and gynes where small <100; medium 100–250; large 251–500; and very large is >501 individuals. Size in parentheses are values reported in the literature for wild colonies. Rearing conditions follow Strange (2015).

mated or unmated. The term foundress queen is used here to distinguish a mother (who founded the colony) from her daughters. Males may be called drones; although we discourage the use of this term for bumblebees as it can create confusion about behavioral traits associated with honeybee drones.

In spring, the mated gyne emerges from the soil, searches for a nesting site, and initiates a new colony. Once a suitable nesting site has been identified, which may take several days or weeks (Free and Butler, 1959), the gyne will begin foraging for pollen and nectar, which she mixes to form a provision mass. Once she accumulates enough provision, she will lay her first clutch of eight to 16 eggs (Goulson, 2010a), and will continue foraging for pollen and nectar as needed to rear this brood into the first generation of workers. During this period, she balances time between foraging and incubating her brood. The solitary phase ends and the social phase begins with the emergence of the first brood of workers (Duchateau and Velthuis, 1988). On average, an egg-laying event will occur seven to 10 days after a suitable nest site has been identified, subsequent brood development lasts three weeks, meaning first workers emerge about four weeks after a gyne identifies a nesting site (Goulson, 2010a).

The social phase includes colony growth and development and the generation of reproductives (males and gynes) and terminates with the death of the colony (Goulson, 2010a; Amsalem et al., 2015). After the first brood emerges, the queen focuses primarily on egg-laying, while the workers perform the nest related tasks of foraging for nectar and pollen, brood care, and cleaning and defending the nest (Goulson, 2010a; Evans et al., 2007; Amsalem et al., 2015). As brood-rearing by workers continues, colony growth accelerates. In some species, such as *Bombus huntii* Greene and *Bombus vosnesenskii* Radoszkowski, the colony can grow rapidly, sometimes reaching 100 individuals after 12 weeks from first oviposition (authors' personal observation; Crone and Williams, 2016). When the colony becomes large enough, a number that varies greatly among species (Free and Butler, 1959), it undergoes a demographic switch to producing reproductive individuals: males and gynes (Cnaani et al., 2002; Goulson, 2010a; Crone and Williams, 2016). Once these reproductives emerge, males remain in the colony only briefly before leaving in search of a mate and never returning (Goulson, 2010a). Unmated gynes will regularly leave the nest to mate and forage for pollen and nectar to fulfill their own nutritional needs, but will frequently return (Alford, 1975; Goulson, 2010a), and in some cases, contribute to provisioning resources for the colony (Allen et al., 1978).

In late summer and early fall, reproductives (males and gynes) are produced by the colony (Sladen, 1912). Fall reproductive individuals will feed on pollen and nectar in the nest for several days following eclosion and gynes may occasionally forage outside the nest before dispersing to mate (Sladen, 1912). Once a newly eclosed gyne has reached sexual maturity, she will leave the nest for the last time to prepare for hibernation (Plath, 1927; Duchateau and Velthuis, 1988). She will continue to feed at flowers and will mate (typically with one male, although multiple mating

has been documented in several species (Plath, 1923; Schmid-Hempel and Schmid-Hempel, 2000) until she identifies a suitable hibernation site. The gyne will excavate a small chamber beneath the soil (*hibernaculum*) where she will diapause for the next six to nine months until the soil warms in spring (Alford, 1969; Heinrich, 2004). These gynes are the only members of the colony to enter hibernation, whereas the foundress queen and her worker and male offspring will die prior to winter. While this constitutes the typical lifecycle for the majority of bumblebee species, many of these features vary among species and across regions (see Sakagami, 1976). For example, reports of *B. impatiens* indicate that mated gynes can actually hibernate together in groups near their natal nest (Plath, 1927). Further, mating behavior, overwintering behavior, and hibernation conditions vary among species and little data exists for most of the roughly 265 described bumblebee species. A captive rearing program for *Bombus* must be designed around the lifecycle of the species being raised in order to maximize success, so understanding the biology of the target species is critical to rearing success (Box 20.1).

20.3 Pathogens, parasites, and pests—an overview

Bumblebees are susceptible to a variety of pests, from viruses to vertebrate predators (Table 20.2, Fig. 20.3). Some nest invaders have a little direct impact on the health of a colony, but many are harmful or deadly and can spread rapidly in wild populations and in a rearing facility. In rearing operations, many of these threats can be eliminated by isolating the bees from the outside world; however, it is imperative to practice proper hygiene (see *General Setup*) in rearing facilities, and to quarantine colonies that might be infected to reduce the risks of problematic outbreaks. A discussion of screening methods for some primary pathogenic infections is included under Sections 20.7 and 20.8.

20.4 Rearing facilities

20.4.1 General setup and equipment

Design and set up a reliable rearing space with sanitary working zones prior to initiating a captive rearing program. Bumblebees are sensitive to noises, vibrations, and movement (Pelletier and McNeil, 2003; Strange, 2010), so designating a separate enclosed space to house the colonies is strongly recommended. Sterilizing the rearing space and any food preparation areas is also advised, particularly if they have been used previously to house bees or other organisms. For most rearing facilities, and certainly, for those rearing numerous colonies simultaneously, open shelves or racks are ideal (Fig. 20.4). If you plan to conduct regular experiments on captive bumblebees, consider a set-up conducive to frequent and/or repeated access to the colonies without the need to move them (such as rearing units laid out on tabletops). In any setup, placing rearing units on moveable trays makes moving colonies easier. Trays are also useful for maintaining hygienic conditions by containing feces and allowing for routine cleaning.

Basic lab equipment (balance, flasks, refrigerator) is needed at various stages of food preparation and rearing. Nectar preparation can be aided by a heated stir plate. Pollen preparation (detailed in Section 20.5) is made easier by using a small food processor to powder the pollen and mix the pollen dough. A net to collect escaped bees, long broad-

BOX 20.1 Pollen-storers versus pocket-makers.

Bumblebee species follow one of two strategies for feeding larvae in the nest. *Pollen storers* store pollen separately from larvae; adult workers then directly feed pollen to individual larvae via regurgitation. *Pocket makers* construct pockets of wax alongside clumps of larvae that the foundress queen, and later foragers, will fill with pollen; the larvae then feed themselves from these provisions. Pollen-storers, which include the major commercialized species *B. terrestris* and *B. impatiens*, are considered easier to rear in captivity. Owing to how pollen is used in the nest, pocket-makers, which include many rare and/or declining species, are generally difficult to establish and maintain in a laboratory setting (Velthuis and van Doorn, 2006; Ptáček et al., 2015). There is growing interest in developing rearing practices for pocket-maker species, both for conservation initiatives (Carnell et al., 2020b) and for pollination of crops for which they might be better suited than are currently commercialized species (Poulsen, 1973; Corbet et al., 1991; Fandiño, 2007). The specific requirements and/or the constraints that are likely to apply when rearing pocket-maker species in captivity are not well understood (see Carnell et al., 2020a,b; Bučánková and Ptáček, 2012). Because rearing success with pocket-making species remains low/uncertain, special attention should be paid to ethical considerations around gyne capture and removal from the wild (see Box 20.4).
See Evans et al. (2007) and Goulson (2010b) for *Bombus* species that fall under each larval feeding strategy.

TABLE 20.2 Some bumblebee pathogens and parasites, with implications to rearing success, detection methods, and potential control measures.

Pathogen/parasite species	Implication(s)	Detection method(s)	Control measure(s)
Family Lipotrophidae			
***Apicystis bombi* (Liu)**	A parasitic alveolate that develops and multiplies in the fat body, degrading it. Screen for this pathogen when gynes die within a few days of capture, or if gynes, ex situ, do not survive overwintering.	Through visual screening of fat body, Malpighian tubules, and contents of hind gut of bumblebees under light microscope at 400X. Polymerase chain reaction of frass or gut contents (Mullins et al., 2020).	Screening of bees brought into facilities. Isolation of infected bees or colonies, monitoring, and sanitation.
Family Trypanosomatidae			
***Crithidia bombi* Lippa & Triggiani** ***Crithidia expoeki* Schmid-Hempel & Tognazzo** ***Lotmaria passim* Schwarz**	Widespread and highly transmissible trypanosomatid parasites that develop in the digestive tract of bumblebees. Screen for these pathogens when reproductive fitness in gynes/queens is negatively impacted.	Through visual screening of fat body, Malpighian tubules, and contents of hind gut of bumblebees under light microscope at 400X. Polymerase chain reaction of frass or gut contents (Tripodi et al., 2018; Mullins et al., 2020).	Screening of bees brought into facilities. Isolation of infected bees or colonies, monitoring, and sanitation.
Family Nosematidae			
***Nosema bombi* Fantham & Porter** ***Nosema ceranae* (Fries et al.)**	A potentially virulent fungal pathogen that infects the gut and Malpighian tubules of bumblebees. Screen for this pathogen when males have low survivorship and swollen abdomens, or when gynes show impaired mating behavior linked to swollen abdomens.	Through visual screening of fat body, Malpighian tubules, and contents of hind gut of bumblebees under light microscope at 400X. Polymerase chain reaction of frass or gut contents (Mullins et al., 2020; Cameron et al., 2011).	Screening of bees brought into facilities. Isolation of infected bees or colonies, monitoring, and sanitation.
Family Sphaerulariidae			
***Sphaerularia bombi* Dufour**	A nematode that infects gynes, effectively sterilizing them. Screen for this pathogen when wild-caught gynes don't start laying eggs. This pathogen cannot complete its lifecycle in closed rearing facilities.	Through visual screening of the abdomen of the bumblebee at dissection at 20–40X.	Removal of wild-caught gynes brought into rearing facilities that do not nest within 21 days. Destruction and proper disposal of parasitized bees.
Family Podapolipidae			
***Locustacarus buchneri* (Stammer)**	An internal mite that infects the respiratory systems (tracheae and air sacs) of infected bumblebees. Consider this infection if captive bees are lethargic and when colonies are permitted to forage outdoors.	Through visual inspection of air sacs and tracheae of the bumblebee at dissection at 20–40X.	Screening of adult bees in facilities. Isolation of infected bees or colonies.
Family Pyralidae			
***Galleria* spp. (wax moths)** ***Plodia interpunctella* (Hübner) (Indian meal moth)**	Moth larvae feed on nectar and pollen in nests. Indian meal moths can retard colony growth through competition for pollen and create a nuisance in the rearing facility.	Through visual inspection of colonies, especially around the brood for larvae and pupae.	Trapping adult moths using pheromone-baited sticky traps. Adding Bt (*Bacillus thuringiensis*) to pollen fed to bees can help control outbreaks (Schesser, 1976)

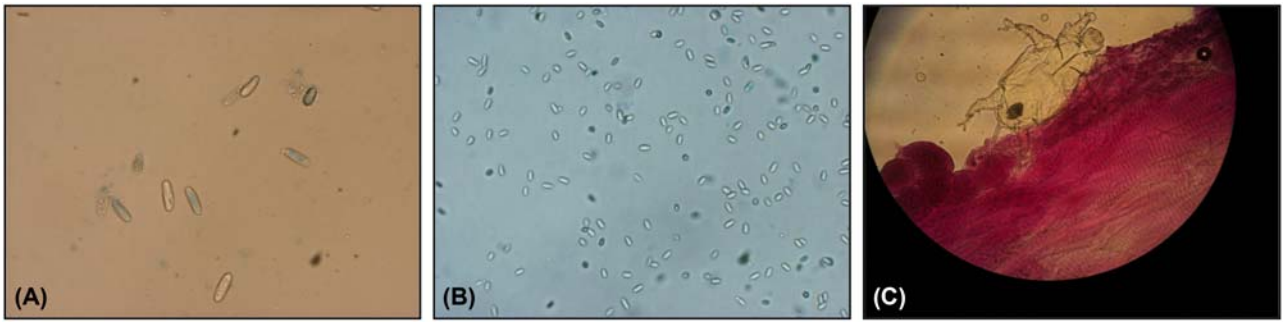

FIGURE 20.3 Phase-contrast microscopy [400X] of cells of the fat body in *Bombus* sp., depicting (A) mature oocysts and spores of *Apicystis bombi*, a parasitic alveolate; and (B) spores of *Nosema bombi*, a fungal microsporidian. (C) An adult *Locustacarus buchneri*, an internal parasitic mite, infecting the trachea and air sacs of *Bombus* sp. [~80X].

FIGURE 20.4 Rearing facility setup at the USDA—ARS: Pollinating Insect-Biology, Management, Systematics Research Unit in Logan, Utah (USA). At this facility, captive bumblebees are placed on trays and shelved on baker's racks within an environmental chamber. The chamber is kept dark at all times, and red light is used sparingly.

tipped forceps for adding and removing contents from the rearing unit (like pollen and bees), and a diluted solution of household bleach (one part household bleach to nine parts water) to sanitize equipment to be used between different colonies are all essential. Some labs steam-sterilize forceps, spatulas, and other tools between use, others use a standard dishwasher on the sanitation cycle.

20.4.2 Environmental conditions

Multiple environmental variables should be considered in a rearing facility, including photoperiod, temperature, and humidity regimes. Photoperiod is known to have an effect on various colony characteristics (Tasei and Aupinel, 1994; Amin et al., 2007a,b,c; Amin and Kwon, 2011) and excessive light may be especially disruptive to gynes upon installation (authors' observations). Twenty-four-hour constant or near-constant darkness has been successful for rearing various wild-caught species (Strange, 2010; Mader et al., 2010; Strange, 2015), and is recommended for most North American species, especially throughout the gyne installation and colony initiation phases. Working with bees under red light minimizes disturbances while yielding enough light to work effectively (Pelletier and McNeil, 2003; Evans et al., 2007; Gurel and Gosterit, 2008). Brief periods of white light exposure do not seem to be highly detrimental, but the amount of light that decreases nesting success has not been determined, so the recommendation is to minimize it.

Maintaining optimal temperature and humidity conditions is critical for successful *Bombus* rearing, and optimal conditions depend on the species and/or the region from which it was collected. In general, the aim is to maintain an ambient temperature between 25°C and 30°C with a relative humidity of 55%−65%; and to refine according to the species (Table 20.3).

TABLE 20.3 Climatic conditions documented in some successful rearing efforts (i.e., workers emerged) for various *Bombus* species.

Bombus species	Temperature (°celsius)	Relative humidity (minimum %)	References
appositus Cresson	28	40–60	Strange (2010)
atratus Franklin	26–28	70	Almanza et al. (2006), Fandiño (2007)
bifarius Cresson	28	40–60	Strange (2010)
borealis Kirby	29	50	Plowright and Jay (1966)
centralis Cresson	28	40–60	Strange (2010)
humilis Illiger	28–30	65–70	Ptáček et al. (2015)
huntii Greene	28	60	Strange (2015)
ignitus (Smith)	27	65	Yoon et al. (2002)
impatiens Cresson	28–30	50	Cnaani et al. (2002)
nevadensis Cresson	29	50	Plowright and Jay (1966)
pascuorum (Scopoli)	28–30	65–70	Ptáček et al. (2015)
perplexus Cresson	29	50	Plowright and Jay (1966)
ruderarius (Müller)	28–30	65–70	Ptáček et al. (2015)
rufocinctus Cresson	29	50	Plowright and Jay (1966)
sylvarum Linnaeus	28–30	65–70	Ptáček et al. (2015)
ternarius Say	29	50	Plowright and Jay (1966)
terrestris (Linnaeus)	28–30	60	Röseler (1985)
	28	60	Yeninar et al. (2000), Gurel and Gosterit (2008)
terricola Kirby	29	50	Plowright and Jay (1966)
vagans Smith	29	50	Plowright and Jay (1966)
vosnesenskii Radoszkowski	28	60	Strange (2015)
	25.5–26.6	60	Malfi et al. (2020)

20.4.3 Bumblebee rearing units

The most convenient and successful rearing unit design for bumblebees will depend on the number of colonies and the species involved. Because colonies can be initiated in small boxes and space can be limiting, most rearing systems involve placing a foundress gyne into a "starter box" or initiation box (Fig. 20.5), and then transferring those that successfully initiate colonies to a larger colony box when workers emerge (Fig. 20.6). These rearing units come in a variety of setups and can be made from a variety of materials (Plowright and Jay, 1966; Evans et al., 2007; Strange, 2010). Herein we refer to the smaller unit used to induce a gyne to found a colony as the initiation box and the larger unit used to house a colony as a colony box. The structure built by the queen and her workers to raise brood we term the nest, whereas the totality of the nest and individual bees comprise the colony. Plowright and Jay (1966) introduced a simple design for wood and wire screen rearing units that are easy to build in a modest woodshop, and Evans et al. (2007) provided instructions for a wooden gyne initiation box. Commercial producers have proprietary initiation and colony boxes made from injection-molded plastic and other materials. Various researchers have also modified off-the-shelf plasticware containers into rearing units (authors' observations). Many designs include specific central areas with an artificial nub that mimics a brood mass and appears to promote broodiness of the gyne. Some rearing groups recommend the addition of cotton (water-repellent upholsterer's cotton; Hobbs et al., 1960) into colony boxes to allow gynes

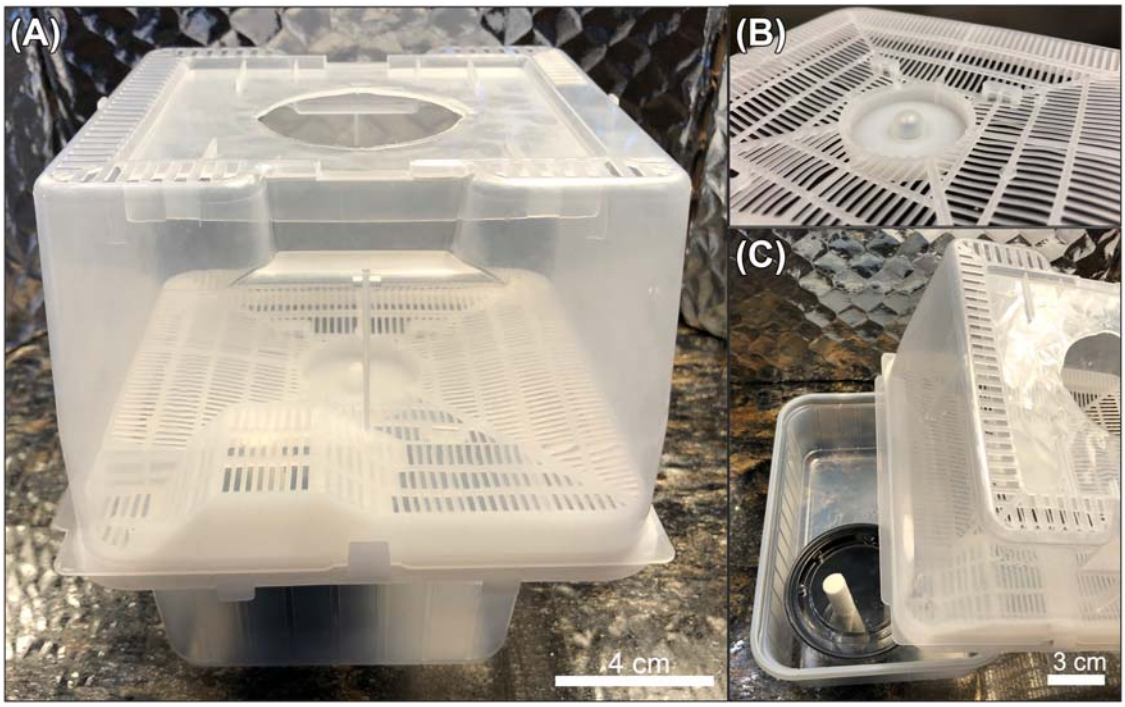

FIGURE 20.5 A Biobest initiation box (A; 15 cm × 15 cm × 10 cm) made from injection-molded plastic. The base of these initiation boxes (B) have an explicit depression (4.5 cm in diameter) in the center where pollen provisions are placed, and an artificial nub that mimics a brood mass and appears to promote broodiness in gynes. In this setup, the initiation box is placed upon a smaller container (C; 18 cm × 12 cm × 5.5 cm) where a wick feeding system (composed of a reservoir for the nectar substitute and a wick reaching to the base of the initiation box where it is accessed by the bumblebees) is held. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

or young colonies to better thermoregulate (Vogt, 1986). Generally, we do not recommend this unless rearing room temperatures cannot be maintained above 26°C as it obscures observation of the developing nest, and removal of the cotton material can often result in the destruction of the nest. If subsequent in-nest monitoring is not required, such material may be beneficial for colonies deployed into field conditions and can be added shortly before deployment.

The feeder system used to supply the nectar substitute will vary with the rearing unit setup options, and these systems can impact the kind of sugar solution that can be used (see *Nutrition*). Some designs involve a wick and reservoir system that sits below the colony (as is the case in most commercial operations), while others involve a gravity-fed system where the nectar substitute is dispensed from an eyedropper, glass tube, or syringe (Evans et al., 2007). Prepared pollen balls (see Section 20.5) are placed within the initiation box, ideally adjacent to the molded artificial nub.

20.5 Nutrition

20.5.1 Nectar substitute

Bumblebees require a near-constant source of carbohydrates (primarily sucrose, with lesser amounts of glucose and fructose; Harder, 1986) to maintain adult activity levels and overall fitness. In nature, bumblebees forage for nectar from flowers and extrafloral nectaries with 15%−50% sugar content (Baker and Delfinado-Baker, 1983; Knopper et al., 2016), but in ex situ settings, they must be supplied with a substitute. Natural nectar varies in sugar content both within and among host plant species, but also in other components, such as amino acids, minerals, and lipids (Baker and Delfinado-Baker, 1983). Artificial nectar solutions vary considerably among *Bombus* rearing facilities, and the quality of the substitute can impact the overall nutritional status and survival rate of bumblebees at various times in the life-cycle (Watrous et al., 2019; Woodard et al., 2019). Various nectar substitutes have proven successful so rearing program objectives and resources should be considered when selecting one.

Simple sucrose solutions (25%−50% sugar) and honey water solutions (1:1) have been commonplace in noncommercial *Bombus* rearing facilities since rearing bumblebees began (Plowright and Jay, 1966). Sucrose solutions, while inexpensive and easy to make, can be nutritionally poor at low concentrations (Watrous et al., 2019) and it can be difficult for bumblebees to associate them as a nectar substitute, often resulting in underconsumption. Honey water solutions are several times more expensive than sugar and carry with them the risk of introducing honeybee (*Apis mellifera* Linnaeus) pathogens into the colonies (Grabowski and Klein, 2017). As such, many facilities are now opting to purchase pre-made, high-quality commercial nectar substitutes, or to develop their own by infusing a high-concentration sucrose solution with preservatives (Box 20.2), feeding stimulants, and additives that help minimize protein stress (Box 20.3). A sugar ratio of approximately 2:1:1 (sucrose: fructose: glucose) with total sugars in solution at 25%−50% provides a solution that will move through a cotton wick and will prevent crystallization. A pure sucrose solution should not be used with wicks as this will crystallize and clog the wick, even at low sugar concentrations, thus invert sugar (see below) which prevents crystallization should be incorporated.

Preservatives, such as sorbic acid potassium salt (see Box 20.2), help in preventing mold and substantially increase the shelf life of a nectar substitute. Note that sorbic acid has low water solubility so use of sorbic acid potassium salt is needed for making the preservative solution. The formulation of the sugar solution provided here has been used for feeding lab bumblebees and can be maintained at room temperature with no mold growth for several weeks. It has been successfully used in 2.0 L feeding reservoirs for two months, both in lab and field conditions. Feeding stimulants are

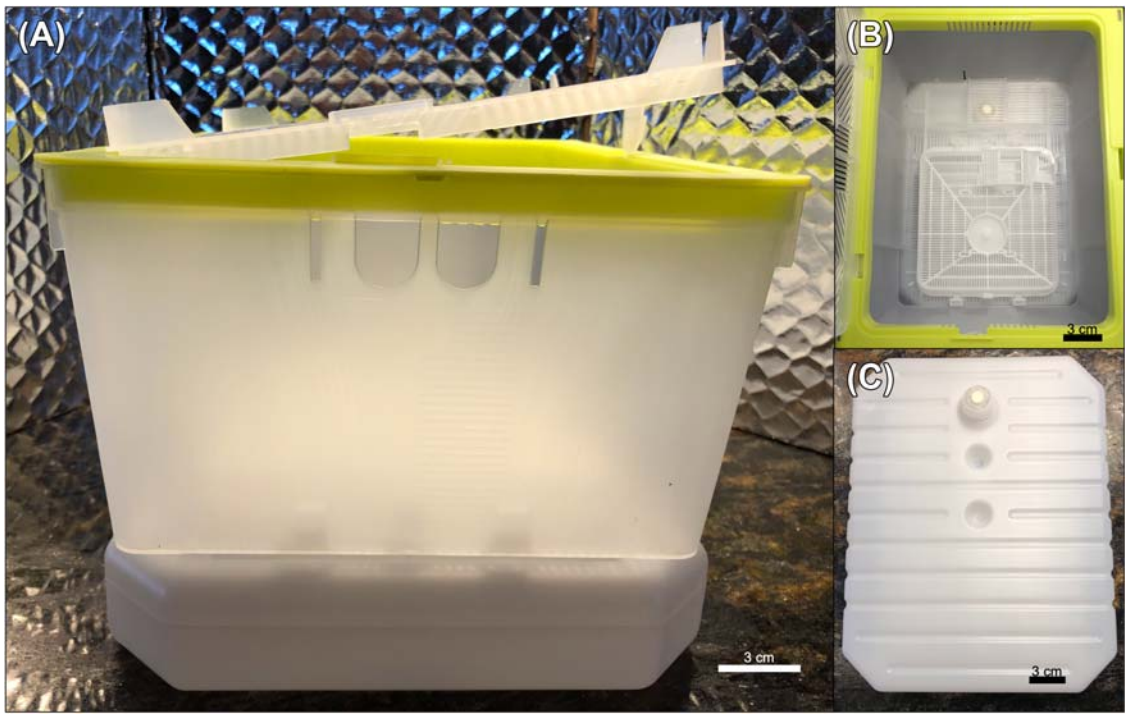

FIGURE 20.6 A Biobest colony box (A; 29 cm × 22 cm × 13 cm) made from injection-molded plastic. These colony boxes are designed to permit free-foraging colonies, but entrance and exit holes on the side can be closed. In this setup, the colony box is placed upon a large nectar reservoir (C; 29 cm × 22 cm × 4.5 cm) containing a wick feeding system (wick visible in both B and C). The base of the initiation box (visible in B) is designed to clip into the base of the colony box when the colony is ready to be transferred.

BOX 20.2 Sorbic acid poatssium salt (sorbic acid) solution (measured by volume).

1. Fill an appropriate bottle with three-parts water.
2. Measure one-part of sorbic acid potassium salt and mix it into the water. Store in a cool, dark place.
3. Use at a rate of 5 mL/L in nectar substitute recipe.

BOX 20.3 Recipe for a cane syrup solution and its invert syrup.

These syrups combine (in equal parts, by volume) to make a high-quality nectar substitute that is relatively easy to prepare and is suitable for feeding to both gynes and growing colonies. This solution was developed at the USDA—ARS: Pollinating Insect-Biology, Management, Systematics Research Unit, Logan, Utah (USA). It has been used in the successful rearing of many *Bombus* species.

Cane Syrup Recipe (yields roughly 2 L).

Ingredients	Instructions
1 L water (preferably distilled)	Combine water and sugar and heat to dissolve the sugar.
1 L table sugar (sucrose)	
9.5 mL sorbic acid solution (Box 20.2)	Once the sugar has fully dissolved, let the solution cool to room temperature.
9.5 mL feeding stimulant	
9.5 mL amino acid supplement	Add the sorbic acid solution, feeding stimulant, and amino acid supplement, and mix well.

Invert Syrup Recipe (yields roughly 2 L)

Ingredients	Instructions
1 L water (preferably distilled)	Combine water and sugar and heat to dissolve the sugar.
1 L table sugar (sucrose)	Bring sugar solution to a boil, then add the citric acid and continue boiling for 20 minutes.
0.95 g citric acid	If the citric acid does not fully dissolve during the boiling process your final solution may crystallize.
9.5 mL sorbic acid solution (Box 20.2)	Remove solution from heat and let cool to room temperature.
9.5 mL feeding stimulant	
9.5 mL amino acid supplement	Add the sorbic acid solution, feeding stimulant, and amino acid supplement, and mix well.

added to help guide bees to the nectar substitute and promote an adequate consumption level to prevent nutritional stress. Honeybee feeding stimulant solutions are commercially available or can be made with essential oils such as spearmint and lemongrass oil added directly to the sugar solution. Amino acids are common constituents of natural nectars but are less commonly added to nectar substitutes. Because they are designed specifically to reduce protein deficiency that can occur as a result of limited pollen availability or low-quality pollen, it is important to consider adding amino acids to nectar substitutes that are supplied to bees in transit, and throughout the life of the colony to increase overall fitness (e.g., aid in stimulating early brood development, building and strengthening weak colonies, and providing additional nutrients for healthy nurse bees). Dietary amino acids and vitamins have been demonstrated to aid the honeybee immune response (Glavinic et al., 2017) and are assumed to be useful for bumblebees as well. Like with feeding stimulants, amino acid solutions marketed for honeybees are commercially available. Mixed nectar substitutes must be refrigerated to prevent degradation and storage guidelines written on the labels of all additives must be followed.

Nectar solutions and their delivery mechanisms within rearing units vary. It is important to consider the type of nectar delivery mechanism when selecting a nectar substitute because the properties of various sugar solutions often impact the efficacy of the delivery mechanism. In the case of high-concentration sucrose solutions, crystallization is known to occur rapidly (Laos et al., 2007) and can impact the ability of the solution to move through a feeder wick, which decreases both the quantity and quality of the solution that is provided. One solution to delay crystallization is to mix an invert sugar with the sucrose solution. The presence of the lower molecular weight monosaccharides (glucose and fructose) in the invert sugar aids in retaining moisture in the solution and delays sucrose crystallization in a supersaturated solution by adsorbing the surface of the sucrose crystals and inhibiting further incorporation of other sucrose molecules (Hartel, 2002). An invert sugar is created through the addition of acid and heat to a sucrose solution (Ranken et al., 1997; and see recipe in Box 20.3). By applying the heat to the acidified sugar solution, the sucrose is hydrolyzed into its component parts, glucose and fructose. Delivery mechanisms should allow the bumblebees easy access to the nectar solution in their rearing unit. Note, open dishes are not a recommended feeding mechanism because bumblebees can become entrapped in the sticky solution and easily drown, and can attract pests such as ants and mice. When using a wick feeding system, ensure the capillary action of the wick is not physically inhibited by pinching or bending it.

20.5.2 Pollen provisions

Bees feed on pollen to obtain proteins, lipids and essential amino acids for larval growth and development, adult health, and reproduction (Vaudo et al., 2018, 2020). Although bumblebees are generalist pollen feeders, they prefer pollen species with higher levels of protein (Hanley et al., 2008; Vaudo et al., 2016b), or with particular protein to lipid ratios (Vaudo et al., 2016a), which benefit overall colony health and fitness (Vanderplanck et al., 2014; Vaudo et al., 2018). Bumblebees in captivity need to be supplied with a constant source of pollen for optimal brood development and reproduction (Kerr et al., 2020).

Pollen for rearing is frequently harvested from honeybee colonies using pollen traps, which collect corbicular loads as workers re-enter the hive following a foraging bout. Because large quantities of pollen can be collected from a honeybee hive (sometimes more than 1 kg/day), this is a relatively reliable and inexpensive source for obtaining adequate amounts. Pollen sourced from commercial operations is often easy to acquire and can be kept frozen for several years, so is usually the most reliable option for bumblebee colonies in the social phase.

The reproductive success of gynes/queens and larval development in bumblebees, however, is often dictated by the quality (protein content and protein to lipid ratio, levels of contamination by pesticides and/or pathogens, and freshness) of pollen provided (Ribeiro et al., 1996; Génissel et al., 2002; Evans et al., 2007; Ptáček et al., 2015). Fresh spring pollen collected from the same location as gyne capture is most palatable to gynes upon installation and appears to improve colony initiation success across many *Bombus* species (authors' observations); however, sourcing this kind of pollen is not without inherent challenges, such as obtaining large quantities from honeybee colonies early in the growing season, from regions where the pesticide landscape is known and floral diversity is high. Upon collection of pollen from honeybee colonies, it is recommended to return the pollen to the rearing facility, remove any nonpollen debris, seal it in vacuum containers, and store it at -20°C. Pollen that is processed on the day of the collection can be stored for later use.

Because pollen is known to contain pathogens common to honeybees and bumblebees (Graystock et al., 2016), some rearing operations choose to sterilize pollen to minimize pest and pathogen transfer. Unfortunately, the sterilization process also kills the beneficial microbial community in the bumble bee's protein source, and there is evidence linking these beneficial pollen-borne microbes to increased overall bee fitness (Dharampal et al., 2019; Fowler et al., 2020; Dharampal et al., 2020). There are several options available to sterilize pollen, each presenting its own costs, risks, and difficulties. The most broadly used method is gamma irradiation; however, chemical methods such as ozone or ethylene oxide fumigation have also been investigated (Gilliam, 1973; Graystock et al., 2016). Sterilization is usually performed after the pollen has been collected and cleaned of debris, but before it is mixed with sugar solution (when outlined in protocols) for feeding to colonies.

20.5.3 Pollen preparation

Honeybee corbicular pollen loads are usually processed further before they are fed to bumblebees. During preparation, disposable lab gloves and clean/sterilized equipment must be used. To prepare the provision, the pollen loads should be ground and mixed with a nectar substitute to create a dough that can be cut into uniform portions and fed to the captive bees. Pollen preparation for portions fed to gynes is more labor-intensive than it is for feeding to colonies. For gynes, 500 mL of unground corbicular pollen loads mixed with approximately 50 mL of nectar solution will yield 50 to 60, 2 g pollen portions. After the pollen is formed into portions it can be dipped in molten organic beeswax to help retain its moisture and nutritive value. This is recommended because it increases brooding success and colony initiation in wild-caught spring gynes (authors' personal observations), in part because it provides additional wax that can be used for constructing honey pots and other parts of the nest and it keeps the pollen lump from drying out.

Preparing Pollen Provisions: For general pollen provision preparation follow steps one to four, and store according to step seven. When preparing pollen provisions for gynes, complete steps one to seven.

1. Measure 500 mL of corbicular pollen loads.
2. In a food processor or using a mortar and pestle, begin by grinding the frozen pollen loads into a powder.
3. To the powdered pollen, slowly add 50 mL of your nectar substitute while the food processor is running or mix with a spoon or spatula in a mixing bowl if you are using a mortar and pestle. Add additional nectar with caution to avoid making the dough too wet. You have an option here to add 10 μL of biological insecticide (98% *Bacillus thuringiensis* subspecies *kurstaki* active ingredient) per 1 mL of nectar to control Indian meal moths [*Plodia interpunctella* (Hübner)] and/or wax moths [*Galleria mellonella* (Linnaeus)].

4. Continue mixing until the consistency of the dough is soft, pliable, and smooth, and can be easily hand molded. Once reached, roll the dough into a cylinder shape until a diameter of 10 mm is reached, and use a razor blade to cut it into 5 mm thick portions (roughly 2 g each).
5. Place beeswax in a heat-safe beaker or pan. Slowly warm the wax until it is liquified, being careful not to burn it or cause excessive smoking.
6. Impale a prepared pollen provision with a pointed probe and dip the pollen patty into the liquid beeswax to coat it. Allow the waxed pollen ball to cool briefly before placing it on a sheet of parchment paper or another clean nonstick surface. Repeat this step using several quartered pollen provisions (see feeding regime under Section 20.7).
7. Store the provisions in an airtight container. Refrigerate for up to two weeks or freeze until needed. Thaw frozen provisions prior feeding gynes.

20.6 Gyne collection and transportation

Collecting gynes from the wild is an integral component of any captive rearing, breeding, or research program. This must be done in spring when gynes are searching for new nests in the wild, and in regions where the target species is known to occur. Because gyne flight times are seasonally limited, the window to collect them from wild populations is generally only a few weeks each year and is directly related to the phenology of the target species within the collection region. It is important to know the expected emergence time of the target species in the specific region where you intend to collect gynes. For information on the phenology of North American bumblebee fauna, see Williams et al. (2014).

When determining how many gynes to collect from the wild, consider the impact removing them will have on the wild population (Box 20.4), the intended purpose of creating and maintaining the ex situ population, and rearing facility capacity (which includes adequate personnel for maintaining colonies). It is very important to consider whether a captured gyne has already found a nest in the wild because this can determine population impacts and the likelihood of successful rearing. Gynes that are collecting pollen (have corbicular pollen loads) have most likely begun the process of colony establishment (provisioning for developing brood, oviposition, incubation). The ability of a gyne to revert to these earlier solitary-phase behaviors that define the process of colony establishment varies between *Bombus* species. Unless it is known that the presence of pollen in corbiculae increases nesting success in the lab (see Tripodi and Strange, 2019), collecting pollen-laden gynes should be avoided. If information is not available for the target species, it should be assumed that gynes with pollen have started a nest and their release should be considered. This is especially true for at-risk species (IUCN, 2020).

Capture and transport are inherently stressful for the bees. The negative impact on gyne survival and performance from the time spent in transit can be reduced by careful planning and management of conditions during transport. To minimize the stress experienced by gynes in transit, gynes should be transported individually in small, well-ventilated vials, be provided with access to a high-quality nectar substitute, and be maintained at between 10°C and 12°C to minimize activity. Gynes should be transferred to the ex situ rearing facility and installed in their respective initiation box as soon as possible after collection from the field (a maximum of 4 days from the time of collection will avoid an observable decline in nesting success ex situ). Smaller-bodied gynes within a species also appear to experience higher rates of mortality during shipping relative to larger-bodied gynes (authors' personal observations). It is also worth noting that some nonlethal sampling methods for obtaining genetic material, at least when conducted on gynes, may have a negative impact on their survival (like tarsal clipping, Mola et al., 2021).

BOX 20.4 Some ethics behind *Bombus* collection from wild populations.

The collection of bumblebees from wild populations to support the establishment of commercial rearing facilities, scientific research, and targeted conservation initiatives is not without an ethical dilemma. Early commercial bumblebee programs were built on the removal of tens of thousands of gynes from wild populations (Velthuis and van Doorn, 2006), and in today's climate where global increases in bumblebee declines are reported annually, there is a genuine conservation concern over robbing nature of gynes. Removing foundress queens, those that show evidence of colony establishment, from wild populations results in the death of the colony and/or the loss of the primary reproductive unit in the population (worker bumblebees are unable to mate or lay fertilized eggs). This has obvious repercussions for overall population dynamics because gyne survival and dispersal dictate the distribution of bumblebee nests across the landscape. Wild collection of gynes should follow an in-depth consideration of the impact their removal could have on the population in that region, and these considerations should inform reasonable yields prior to any collection efforts.

20.7 Installing gynes and stimulating broodiness

Each gyne should be installed in an initiation box with a high-quality nectar substitute and a wax-coated pollen ball (see recipes and preparation methods under Section 20.5, and box design under Section 20.4.1) placed adjacent to the artificial brood (nub) when present. Install gynes as soon as possible after collecting them from the wild and leave them undisturbed for a period of 48–72 hours. After this settling period, gynes should be fed a smaller waxed spring pollen ball (roughly ¼ of a full pollen ball) placed adjacent to the original pollen ball every 48 hours. The original pollen ball should only be removed if the gyne has not oviposited or demonstrated incubation behavior for a minimum of seven days postinstallation and can remain in the box indefinitely. The nectar substitute should always be available ad libitum.

Stimulating broodiness (behaviors associated with egg-laying; see Gamboa, Foster, and Richards, 1987; and Cameron, 1989 for details) in wild-caught gynes is critical to any rearing program, but it is arguably one of the most difficult tasks (Box 20.5). Many techniques to increase broodiness have been trialed (Kwon et al., 2003), but few show consistent promising results (Kwon et al., 2006), and variation of nesting success across species points to the need to tailor nesting stimulators to individual taxa (Plowright and Jay, 1966; Mah et al., 2001; Kwon et al., 2006; Yoneda, 2008; Strange, 2010). The most commonly used techniques today are: (1) pairing conspecific gynes in a single initiation box; (2) introducing callow honeybee or bumblebee workers; (3) introducing comb/brood, larva, or pupa;(4) aggressive CO_2 narcosis. The suitability of each is largely based on individual research objectives and rearing programs.

Pairing wild-caught spring gynes in a single initiation box have resulted in higher colony initiation success, but it can result in one gyne killing the other (Sladen, 1912; Duchateau, 1991; Van den Eijnde et al., 1991; Ptáček et al., 2000; Strange, 2010). Alternatively, gynes can be installed alongside two or more callow worker bees (*Apis* or *Bombus*) (Sladen, 1912; Ptacek, 1985; Duchateau, 1991; Van den Eijnde et al., 1991; Gretenkord and Drescher, 1997; Strange, 2010), or alongside male pupa (Kwon et al., 2003; Kwon et al., 2006). While colony initiation has been improved using these techniques, acquiring callow workers, brood, and/or other life stages all pose their own set of logistic challenges and an increase in the overall risk of disease transfer in the rearing program.

Aggressive CO_2 narcosis is a technique that has been developed to inhibit and control diapause in captive *Bombus* (Röseler, 1985) and has been shown to induce complex pleiotropic effects on gyne physiology that are correlated with an increase in reproductive success and overall fitness (Amsalem and Grozinger, 2017). The technique is common in commercial facilities devoted to mass production and year-round rearing, but it is rarely practiced in programs designed to mimic the natural bumblebee lifecycle, which includes long periods of diapause. With CO_2 narcosis, methods vary according to the duration and schedule of narcosis regimes, and according to the CO_2 concentration in use. In general, a gyne is placed in a tube and flooded with CO_2 until she is unconscious. She is kept in that state for 30 minutes before being placed back into her initiation box. This process is performed either once upon installation before the gyne is left for a settling period, or it is repeated daily until she either oviposits or dies (Röseler, 1985; Velthuis and van Doorn, 2006; Gosterit and Gurel, 2009). This technique is rarely recommended because the methods behind it are labor-intensive and only vaguely described and because its capacity to initiate broodiness in bumblebee gynes has not been consistently demonstrated.

20.8 Colony care and senescence

Once a queen has successfully initiated a colony, the emergence of her first brood occurs 3–4 weeks after the first oviposition (e.g., average of 3 weeks for *B. terrestris*, Duchateau and Velthuis, 1988), but timing can be affected by temperature, the quality and availability of pollen and nectar, and species-specific life histories (Kerr et al., 2020). Maintaining the optimal environment (see Section 20.4.1) and supplying adequate amounts of high-quality pollen (see

BOX 20.5 A note on nonproductive gynes.

Securing a large number of gynes to start a captive population can be difficult and maintaining them is labor-intensive. It may be necessary to cull or release nonproductive gynes (those without brood) as nesting success is nearly always less than 100%. Because most gynes that become successful at producing large colonies initiate a nest within 21 days after installation, culling the gynes at 21 days is recommended if no brood is present. If using CO_2 narcosis protocols to promote broodiness, you may consider extending this period, but successful nest initiation is not expected from nonproductive gynes after 21 days postinstallation.

Section 20.5) are essential to prevent aborted larval development during this phase (usually evident when the queen removes developing larvae from the wax brood capsules).

Following the emergence of workers in the first brood, the social phase of the lifecycle begins, and the growing colony should be moved from the initiation box to a larger colony box (see Section 20.4.1). This transfer should be done under red light, and according to the dimensions of your initiation box. When initiation boxes are small (smaller than 10 cm × 10 cm × 8 cm), the transfer should occur after the emergence of the first brood (roughly five workers) when the colony is small enough to manage but large enough to be resilient to the disturbance. When initiation boxes are larger (12 cm × 12 cm × 8 cm, a standard Biobest initiation box), then transfer to a larger colony box can be delayed until after the second brood of workers emerges (10−15 workers). Fresh nectar substitute needs to be always available to captive bumblebees, and a fresh pollen ball will usually need to be supplied every other day but the amount and frequency of the feeding of pollen provisions will increase as the colony grows. There is anecdotal evidence suggesting that too much pollen supplied to a colony in the social phase can lead to early gyne production, thereby impacting colony growth and reproductive potential, and can encourage the growth of mold and/or support unwanted moth infestations. Additional pollen should only be fed once daily to colonies that have fully consumed the pollen portion(s) that have been supplied previously.

When the colony reaches a peak size, it undergoes a demographic transition ("switch point") and begins producing reproductive individuals (second-generation queens, known as *gynes*, and males) rather than workers (Goulson, 2010a; Amsalem et al., 2015). In some species, occasional males are produced early, but the switch point represents a clear transition away from worker production. For robust colonies, the switch is first signaled by the presence of very large brood cells containing gyne larvae and pupae. Males emerge first (from brood cells that are the same size as worker cells), followed soon after by new gynes. Colonies kept in the lab through the full life cycle will die off naturally in four to six months after the first oviposition. All rearing equipment should be cleaned thoroughly between generations. After scraping excess pollen, wax, and cocoons from the nest, wash in warm water and then soak all rearing components in a one part household bleach to nine parts water solution and air dry it fully before storing it. Plastic rearing supplies and equipment can be cleaned in a dishwasher on a sanitizing cycle (Box 20.6).

20.8.1 Sanitation

Caring for captive colonies is laborious. In addition to ensuring that the nutritional needs of the bumblebees are met through feeding, meeting the sanitation requirements in a rearing facility can be challenging. Colonies need to be inspected regularly for dead bees and ejected larvae, which should be removed from the rearing unit upon discovery. In instances where dramatic, sudden population drops are observed in a colony, isolation of the colony must follow; this could signal the presence of disease.

BOX 20.6 Microcolonies.

Microcolonies are small groups of bumblebee workers (typically three to five) separated from their natal colony and reared in isolation of a queen. Through separation from the queen, a dominant worker is stimulated to begin laying unfertilized eggs (Free, 1955) that will develop into males. The microcolony will forage for and tend these offspring. Microcolonies allow for greater replication for experimental use, permitting much higher sample sizes than would be feasible using queen-right colonies. The use of microcolonies in *Bombus* work is thus a powerful tool for addressing broad experimental questions in behavior, development, microbiome, pesticide assessment, and social evolution (reviewed in Klinger et al., 2019).

Research objectives dictate exact protocols for rearing *Bombus* microcolonies. Generally, they can be initiated both from callow (newly emerged) workers or from workers of unknown age, with the former suggested as best for investigating the effects of experimental treatments, and the latter for producing males for reproduction or for creating pathogen cultures (Klinger et al., 2019). Colonies from which workers are sourced for microcolonies should be in a growing phase, producing new workers daily, but not yet producing males as this often signals a switch to slow or cease worker production and removing workers at this time could be taxing on the colony.

Microcolonies have been successfully reared in wood, plastic, Styrofoam, and metal rearing units, and the utility of each has been discussed in experimental and logistical contexts (Klinger et al., 2019). The suitability of methods for various species is not well understood, but it is clear that the ease of creating microcolonies varies among them. Regardless of specific protocols (rearing unit, experimental treatments, etc.), microcolonies need access to pollen and nectar and need to be maintained under the same conditions (light, temperature, and humidity) as would any queen-right colony.

Developing a sanitation or "clean stock" plan prior to rearing is important. This should include a plan for prevention, detection, treatment, and elimination of diseases that may occur in the rearing facility. As mentioned previously, cultural control of pests is the most effective strategy. This can include sterilizing equipment regularly, monitoring and trapping pests, and physical barriers such as screening to prevent pest infestations. Detection protocols can include colony inspections, the use of traps, and microscopy or PCR detection of pathogens. Whereas chemical control of many diseases is not available, some do exist and physical measures such as colony quarantine and isolation can be employed when other effective treatments are lacking. Finally, elimination of diseases and pests should be the ultimate goal, and combined monitoring, treatment, and isolation strategies should be employed to reach the goal of clean bumblebee stock.

20.8.2 Deploying colonies into the wild

Before releasing bees from a captive rearing program into a wild setting, and before allowing captive bees to forage outdoors, the impact that this would have on wild populations needs to be carefully considered. Wild bees around the world, including bumblebees, have suffered from the emergence of novel pathogens and the proliferation of disease and parasites owing to the importation and use of managed bees (reviewed by Graystock et al., 2015; Cameron et al., 2016), including recent instances where exotic species have been introduced to new regions (Goka et al., 2001; Sachman-Ruiz et al., 2015). Most of this impact has been the result of commercial industries, but with growing numbers of research laboratories and conservation programs centered around rearing bumblebees (either from purchased colonies or initiated from wild-caught gynes), the same measures must be taken by these facilities to prevent negatively impacting wild bumblebee populations. Colonies should be visually inspected for common pathogens and parasites before being deployed, molecular screening should be considered as resources allow, and caution should be taken not to deploy colonies to regions where they were not originally collected, even if those regions are within the species' native range.

Apart from considering the potential impact colony deployment can have on wild populations, efforts to maximize the likelihood of survival in the deployed bumblebees should also be made. In the wild, bumblebees most commonly nest underground, but colony deployment from an ex situ setting almost always occurs above ground. This mismatch often leads to an inability to thermoregulate within the nest, so it is important to ensure the colony is placed in an area that provides sufficient shade during the day, and it is often recommended to insulate the colony with upholsterer's cotton (Hobbs et al., 1960; Williams et al., 2012; Malfi et al., 2020).

20.9 Mating trials

Bumblebee mating varies greatly across the genus, and optimal mating strategies for most species are not fully understood. Assessing *Bombus* mating behavior can be difficult and mating trials frequently yield low success in captivity. Despite advancements in our understanding of mating strategies, we still know very little about the visual, behavioral, and olfactory cues that various *Bombus* species use during mating (Velthuis, 2002; Van Honk et al., 1978; Alford, 1975; Bergström, 1982). The mating period is brief in the bumblebee life cycle, and in nature, it takes place when the gynes and males leave their natal nests. The foundation of many present-day mating methods was based on Röseler (1985) who published methods developed for *B. terrestris*. In noncommercial facilities currently rearing *Bombus*, the success of mating trials is low, but for some species, there appear to be ways to improve the chances of success.

Maintaining a consistent schedule of pairing gynes and males based on sexual maturity is thought to improve the compatibility of mates by synching their mating biology and behavior (Herndon, 2020; Tasei et al., 1998; Djegham et al., 1994). To implement this, when a colony has matured and begins producing reproductives, newly emerged gynes and males should be removed every 48 hours. Gynes and males must be housed in separate rearing containers once they have been removed from the colony, but gynes can be coupled with other gynes removed on the same day, and similarly for males. Feed pollen and nectar substitute ad libitum until males have reached sexual maturity (roughly seven to nine days after emergence), and mature ovaries have been developed in gynes (roughly nine to 11 days after emergence) (Tasei et al., 1998; Kwon et al., 2006; Jung et al., 2001; Lee et al., 2002; Amin et al., 2011; Herndon, 2020).

Reproductives should not undergo mating trials before a period of two days has elapsed from the time they were removed from their natal colonies. Because males can reach sexual maturity before ovarian development is complete in gynes, they should be paired with younger gynes (Amin and Kwon, 2011). Reproductives should be placed into mating cages with a male to gyne ratio of 2:1 (Amin et al., 2010; Kwon et al., 2006). Photoperiodic regimes have been shown to influence mating success in some species (such as *B. terrestris*, Amin and Kwon, 2011), and anecdotal evidence

suggests that most bumblebee species preferentially mate between 7 a.m. and 11 a.m., and at dusk. Nevertheless, mating can occur at any time, and the scheduling demand of continuous observations can make copulation difficult to record.

Many sizes and shapes of mating arenas have been trialed (BOX 20.7). Selection of the arena's design should consider research needs as some can impair the ability to make observations with ease, can limit access to individuals, or do not provide the space or ventilation required by some species. Bumblebees in mating trials require access to pollen and nectar ad libitum.

A successful mating event is usually very apparent in bumblebees while it is occurring. During copulation, the male will position himself by holding the gyne's thorax, after which the receptive gyne will extend her stinger to facilitate insertion of the male genitalia (Ings et al., 2005). Once copulation begins, the pair can be removed from the mating arena and placed separately in another small rearing unit, such as an initiation box, until mating has ended. This step will better enable keeping track of which gynes have mated, and how long that mating event lasted. The duration of copulation is anywhere from ten to 40 minutes (Amin et al., 2009; Duvoisin et al., 1999). Gynes that fail to mate within 13 days of emergence are unlikely to do so (Kwon et al., 2006; Jung et al., 2001; Lee et al., 2002).

20.10 Overwintering gynes

Despite many researchers attempting various overwintering protocols, cold storage is still usually associated with high mortality rates, even when the duration of exposure to cold storage is short relative to the length of time they would spend in this period of diapause in the wild (Holm, 1962, 1972; Lindsay, 2020). Attempts to overwinter North American *Bombus* spp. gynes beyond three months have been met with low success rates. Ongoing studies are investigating the physiological costs associated with overwintering variables (Lindsay, 2020; Treanore et al., 2020), hoping to inform new methods of controlled cold storage that can support rearing programs designed to run from one year to the next. Currently, the most common method adopted by *Bombus* rearing facilities is artificial hibernation of mated gynes at a constant temperature, as outlined in Table 20.4.

20.11 Closing remarks

Captive rearing has played an important role informing fields as diverse as animal behavior, environmental toxicology, pollination biology, invertebrate pathology, and social evolution. Despite this, our understanding of the basic biology and ecology of most bumblebee species remains limited. The use of ex situ bumblebee colonies (developed from both wild-caught and laboratory-produced gynes) for scientific investigations has great potential for addressing knowledge gaps in these and other fields, but the practice comes with some caveats.

Traditionally, bumblebees have been mass reared for commercial pollination services. Multiple species have been distributed for pollination of crops such as tomatoes, peppers, berry and fruit crops, and tree nuts. The ability to provide thousands of bumblebee colonies for greenhouse crop production is critical to year-round vegetable availability. However, the potential to use captive mass rearing techniques for bumblebees is now being explored in earnest by bumblebee biologists, ecologists, and conservation practitioners.

Captive rearing offers a unique opportunity to design comparative studies to explore major knowledge gaps ex situ, but concerns around introducing novel traits selected by the commercial rearing of bumblebees are growing. *Bombus* researchers are becoming more adept at locating bumblebee nests in the wild and are recognizing that the ecological risks associated with current management practices require consideration. There are benefits to conducting both ex situ experiments and in situ observational experiments, and the risks associated with each need to be weighed in any research or conservation initiative.

BOX 20.7 Mating arenas.

Mesh enclosures are the most common mating arenas. They range in size from small table-top insect habitats to large outdoor gazebo-like structures designed to promote flight. Most can be sourced and purchased with ease, and smaller options can be relocated to support various research objectives. Glass terrariums have been used to maximize ease of observation, but they come with size limitations and can have inadequate ventilation to support longer trials. Some common species (*Bombus impatiens*) have successfully mated in small plastic arenas (initiation boxes), but the mating behavior of other species (*Bombus griseocollis*) may require large arenas for flight.

TABLE 20.4 Some protocols for overwintering mated gynes, ex situ.

Phase	Elapsed time (hours) post-mating			
	0	24	48	72
Allow gynes to forage on pollen and nectar individually in a small rearing unit, such as an initiation box. Starting weight has been positively correlated with overwintering success since captive rearing began. This initial foraging phase should not be less than 48 hours.				
Place gynes into a temporary holding area at 8°C. The benefits of this phase have not been formally demonstrated, but some consider this stepwise cool down to a final overwintering temperature to be an effective way to reduce physiological stress or shock in gynes.				
Transfer gynes into small individual overwintering units,[a] and label them appropriately. These overwintering units can be stacked on top of one another in the overwintering chamber, but ensure they lie flat.				
Place individual overwintering units in the overwintering chamber. Overwintering chambers are usually a reach-in incubator. Designate the overwintering chamber to maintain the desired overwintering temperature (usually 4°C, but overwintering temperatures can vary with species and experimental needs) and relative humidity of 60% to 70% (adjust with saline solution as needed). When you are ready to have gynes initiate a colony, remove the gynes from the overwintering chamber, install them in initiation boxes, and begin attempting to induce broodiness (see *Installing Gynes and Promoting Broodiness*).				

Notes: Individual research objectives will ultimately dictate the design of overwintering trials. Very few overwintering protocols are created equal. At a minimum, record the date and time when a gyne begins and ends each phase, and her weight at each of those times.
[a]*Individual gynes can be placed in small, clean containers, such as cardboard boxes (8.3 cm × 3.6 cm × 3.6 cm) or plastic vials that have some ventilation.*

Captive colonies of bumblebee species, especially at-risk species, have great potential for conservation-restoration in locations where those species are in decline or have been extirpated. The use of locally sourced species for pollination of crops provides us with a tool to reduce the risk of introducing nonnative species, novel pathogens, and nonlocal genotypes to wild populations. Conservation organizations and *Bombus* researchers have suggested that expanding the use of locally sourced bumblebees for pollination is important both for promoting the health of wild bee populations and for food security. However, further development of rearing techniques, a better understanding of the biology of those individual species which we hope to conserve, and more knowledge about the landscapes from which they originate is required to ensure success.

Bumblebee rearing in captivity can be quite challenging. Many of the protocols for ex situ rearing of bumblebees remain proprietary information and, in many cases, do not translate well to rearing novel species, especially when the goal is to mimic the natural timing of the bumblebee lifecycle. Various rearing protocols currently being implemented utilize insight gained from prior commercial and experimental bumblebee rearing. And, while difficulties persist, there are many scenarios wherein ex situ bumblebee rearing have been fruitful. Yet, the utility and success of maintaining prolific bumblebee research and conservation programs will depend on the continuous development of sound and standardized methodologies. Advancing our understanding of bumblebee biology and ecology will depend on standardizing captive rearing techniques and ensuring that transparency and accessibility are maintained across rearing programs and the *Bombus* community.

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Chapter 21

Current and potential benefits of mass earthworm culture

Christopher N. Lowe[1], Kevin R. Butt[1] and Rhonda L. Sherman[2]
[1]*Earthworm Research Group, University of Central Lancashire, Preston, United Kingdom,* [2]*Department of Horticulture Science, North Carolina State University, Raleigh, NC, United States*

21.1 Introduction

Earthworms form a major component of the fauna of fertile soils. The importance of earthworms in improving crop yields and maintaining soil fertility has been recognized for millennia. In Ancient Greece, Aristotle referred to earthworms as "the intestines of the earth" and during the reign of Cleopatra in early Egypt, the earthworm was considered a sacred animal, with removal from the soil strictly forbidden (Brown et al., 2003). More recently, White (1789) described the importance of earthworms in perforating and loosening the soil, increasing fertility by dragging in leaves and casting them on the soil surface (Cadée, 2003). However, Charles Darwin (1881) is more widely acknowledged (if incorrectly) as the first to recognize the importance of earthworms in soil formation, referring to "these lowly organized creatures" as "nature's plough" (Darwin, 1881).

Today, earthworms are considered to be "ecosystem engineers" (Lavelle et al., 1997) able to regulate natural processes, to an extent that overrides organisms in other functional categories and modify soil function and ecosystem services (Blouin et al., 2013). Earthworms play a major role in improving the aeration, drainage and water holding capacity of the soil. Through their actions, organic material, such as leaves and crop residues, are incorporated into the mineral soil, increasing the availability of nutrients for plants and other soil fauna. Aside from improving soil fertility, earthworms are also a source of protein for many invertebrates like carabid beetles (Harper et al., 2005) and vertebrate species such as badgers (Kruuk, 1978) and moles (Raw, 1966) and even form a part in the diet of some tropical, indigenous human populations (Paoletti et al., 2003). Earthworms are also utilized by humans as a food source for farm animals, including chickens and pigs (Kostecka and Pączka, 2006), as pet food, such as a dietary supplement for ornamental fish and as bait for recreational fishing (Tomlin, 1983; Steckley, 2020).

Earthworms also have a role in several applied areas including land restoration (Butt, et al., 1997), and organic waste recycling (Fernández-Gómez et al., 2010; Sherman, 2018), and ecotoxicology (Byung-Tae et al., 2008). A demand for earthworms, therefore, exists to satisfy scientific and commercial applications. This has resulted in the development of commercial, often large-scale, earthworm collection, storage and transportation industries in North America (Steckley, 2020), predominantly for the established fishing bait market, and the breeding of earthworms (referred to as vermiculture) which forms the focus of this chapter.

Sabine (1988) suggested that vermiculture could be categorized as either low or high technology. Low technology implies small-scale, individual or community level activity, often in a developing country, that seeks to maximize biological resource use such as (vermi) composting of organic residues with little financial input or gain. In contrast, high technology vermiculture implies an industrial-scale, commercial approach with high financial input (often associated with risk) but also a high degree of scientific interest. Before embarking on the details of earthworm culture, there is a need to establish some basic information relating to earthworm ecology.

21.1.1 Ecological groupings

Globally, some 6000 earthworm species have been described, existing under a range of climatic regimes from boreal to tropical biomes (Butt and Lowe, 2011). All earthworms have a similar life cycle. After a period of weeks to months, an

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earthworm cocoon will hatch in the soil or leaf litter to produce a hatchling worm. This animal is free-living and grows over a period of weeks or months to maturity, shown by the presence of a clitellum (saddle). Earthworms are hermaphrodites (act as both male and female), but most species reproduce sexually, by exchange of sperm (amphimictic), whilst others reproduce asexually (parthenogenetic). Cocoons are produced periodically by adults through secretions from the clitellum and deposited in the soil (see Fig. 21.1). Exact timescales and numbers produced are species specific.

Earthworm communities are separated into ecological niches by vertical and horizontal spatial distributions, feeding specialization and species size (Lavelle, 1983). It is therefore possible to subdivide earthworms into categories based upon these ecological and physical characteristics. Bouché (1972) identified three major ecological categories among earthworms and proposed the widely accepted terminology of anecic, endogeic and epigeic (Table 21.1). More recently, further ecological subcategories have been proposed. This is exemplified by anecic earthworms being subdivided into epi-anecic and strict-anecic with the former preferentially consuming fresh litter at the soil surface and the latter a mix of leaf litter and humified organic matter (OM) in the soil profile (Hoeffner et al., 2019). For the purposes of this chapter, it is more relevant to separate earthworms into two distinct categories—those that inhabit OM (litter/compost dwelling) and those that inhabit the mineral soil (soil-dwelling).

21.1.2 Selection of species

A relatively small number of earthworm species have been successfully cultured for scientific or commercial purposes. Vermiculture has often concentrated on culturing a restricted number of litter-dwelling species [e.g., *Eisenia fetida* (Savigny), *Dendrodrilus rubidus* (Savigny), *Dendrobaena veneta* (Rosa), *Lumbricus rubellus* (Hoffmeister), *Eudrilus eugeniae* (Kinberg), *Perionyx excavatus* (Perrier), and *Pheretima elongata* (Perrier)] that have commercial applications (like processing organic residues into a potentially saleable product). Litter-dwelling species are ideally suited to large-scale breeding programs due to high growth, maturation and reproductive rates. In addition, these species can be relatively easily maintained at high densities in organic media (in the absence of mineral soil). Once processed, these organic media may also have a commercial value (as a soil amendment). As a result, there has been significant research effort focused on the culture and maintenance of these species (Edwards et al., 2011), which has led to the adoption of litter-dwelling species (often without justification) in emerging fields of earthworm research such as ecotoxicology (Section 21.2.7). The culture of soil-dwelling (endogeic and anecic) species has been almost exclusively restricted to scientific studies (Lowe and Butt, 2005) with commercial availability only achievable through field collection. It is widely accepted that lengthy life cycles, low fecundity and a requirement for an appropriate soil-based culture medium do not predispose these species to large-scale breeding programs. However, potential commercial applications (such as in land restoration) and an acknowledgment of their ecological relevance (compared with litter dwellers) in environmental monitoring and ecotoxicology have seen an increase in demand for soil-dwelling earthworms of known provenance.

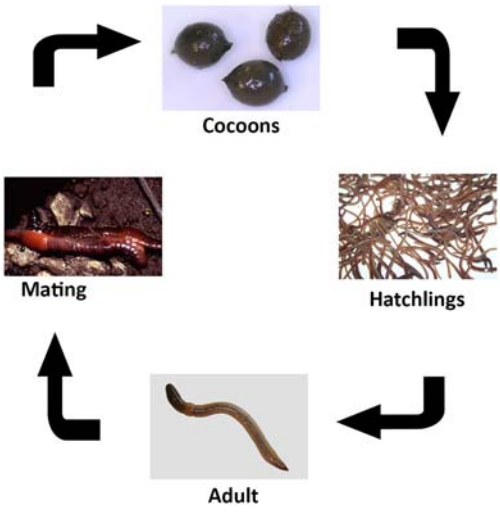

FIGURE 21.1 Stylized life cycle of an amphimictic earthworm—*Lumbricus terrestris* used for illustration.

TABLE 21.1 Generalized characteristics of the three earthworm ecological groupings proposed by Bouché (1972), including temperate and tropical species examples.

Characteristic	Epigeic	Endogeic	Anecic
Adult size	Small-medium	Medium	Large
Reproductive rate	High	Intermediate	Low
Longevity	Short-lived	Intermediate	Long-lived
Pigmentation	Predominantly red colouration	Predominantly unpigmented	Predominantly brown to black colouration
Mobility	Highly mobile—selected members of this group are considered pioneer species	Intermediate	Low mobility, generally found in undisturbed habitats, inhabiting semipermanent burrows.
Location in the soil profile	Organic horizons in or near the surface litter	Usually found in the soil profile inhabiting horizontal burrows within 15 cm of the surface (in temperate locations)	Construct semipermanent vertical burrows (to a depth of 2 m) in the soil profile. Burrows open onto the soil surface.
Food source	Decomposing surface litter	Organic matter ingested with soil (geophagous)	Surface litter drawn into the burrow—usually at night
Temperate examples	*Eisenia fetida, Dendrodrilus rubidus*	*Aporrectodea caliginosa Aporrectodea rosea*	*Aporrectodea longa Lumbricus terrestris*
Tropical examples	*Perionyx excavates Eudrilius eugeniae*	*Pontoscolex corethrurus Millsonia anomala*	*Amynthas rodericensis Eutyphoeus gammiei*

Source: Adapted from Butt, K.R., Lowe, C.N. (2011). Controlled cultivation of endogeic and anecic earthworms. In: Karaca, A. Biology of Earthworms, Springer-Verlag, Berlin, Heidleberg, pp 107–122.

21.1.3 Cultivation techniques

The primary aim of all earthworm cultivation techniques is to develop a sustainable population while at the same time maximizing output. To achieve this goal requires the maintenance of environmental conditions (abiotic and biotic) optimal for earthworm cocoon production, development and growth. Optimal culture conditions are species-specific and are predominantly influenced by ecological grouping (Table 21.2). Furthermore, earthworm culture can be labor intensive due to the requirement for the separation of all life stages from the culture medium. This has led to the development of semiautomated systems for the large-scale production of litter-dwelling species and is considered the key factor currently restricting the commercial culture of soil-dwelling species and continued reliance on field collection.

Significant advances in the development of culture techniques for temperate soil-dwelling, and earthworm species have been made in the last 30 years (Lowe and Butt, 2005). This body of research has demonstrated the feasibility of producing sustainable cultures of both anecic and endogeic species for relatively small-scale use in scientific studies (Section 21.2.6). Nevertheless, the potential development of commercially viable, ex situ, large-scale production of soil-dwelling species is a significant challenge. A more feasible alternative may be in situ encouragement of naturally occurring populations, a concept referred to as biostimulation (Brun et al., 1987) (see Section 21.3).

21.2 Current applications

21.2.1 As a protein source

Throughout the world, earthworms are used as a protein source for animals and humans. Earthworms are fed to farm stock, poultry, pets (including rodents, amphibians, reptiles and fish) and zoo animals. Lawrence and Millar (1945) were the first to suggest that earthworms could be used in commercial feed for animals. Earthworm meal is currently sold for animal feed by companies across the globe but in the United States, it is not approved by the American

TABLE 21.2 Selected life cycle characteristics and culture requirements for selected epigeic, anecic, and endogeic species.

	Epigeic		Endogeic		Anecic	
	Eisenia fetida	*Eudrilus eugeniae*	*Allolobophora chlorotica*	*Aporrectodea caliginosa*	*Aporrectodea longa*	*Lumbricus terrestris*
Mode of reproduction	Parthenogenetic	Amphimictic	Amphimictic	Amphimictic	Amphimictic	Amphimictic
Growth to maturity (days)	28–30	40–49	84 (at 15°C)	No data available	168 (at 15°C)	112 (at 15°C)
Cocoon incubation (days)	18–26	12–16	51–59 (at 15°C)	62–84 (at 15°C)	54–61(at 15°C)	90 (at 15°C)
Cocoon viability (%)	73–80	75–84	62 (at 15°C)	90 (at 20°C)	70 (at 15°C)	83 (at 15°C)
Number of cocoons (day^{-1})	0.35–0.5	0.42–0.51	0.05 (at 15°C)	0.08 (at 15°C)	0.05 (at 20°C)	0.1 (at 15°C)
Optimal moisture content (%)	80–85	80	25	25	25	25
Optimal temperature (°C)	25	25	15	15	15	15

Source: Adapted from Lowe and Butt (2005), Butt and Lowe (2011), and Domínguez (2004).

Association of Feed Control Officials as an ingredient of commercial animal feed. Individuals can raise earthworms and feed them to their own animals, but earthworms cannot be sold as animal feed ingredients.

McInroy (1971) conducted the first compositional analysis of earthworm tissues, concluding that nutrient contents are suitable for both animal and human diets. Dry matter protein content has been reported in the range of 54.6%–71.0% (Sun et al., 1997; Kangmin, 2005; Edwards and Niederer, 1988) and research has indicated that the mean values for essential amino acids fall within the range recommended by the Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO). Compared with commonly consumed human foods and animal feed, earthworm protein and amino acid structures are like those recorded in fishmeal and chicken eggs and higher than cow milk powder and soybean meal (Sun et al., 1997). Earthworm tissues also contain a wide range of essential vitamins. Kangmin (2005) reported that earthworms are rich in vitamins A and B, with 100 g of earthworms containing 0.25 mg of vitamin B1 and 2.3 mg of vitamin B2, with vitamin D comprising up to 0.07% of earthworm wet weight. In addition, Sun et al. (1997) suggested that earthworm castings have the potential to partially replace cornmeal or wheat bran in animal feed as *E. fetida* casts contained protein levels (7.9% dry matter) comparable to cornmeal. These researchers also reported that the body fluids of earthworms contained high amounts of vitamins and minerals (especially iron), 9.4% protein and 78.8% free amino acids per liter.

Sabine (1978) conducted the first animal feeding trial using earthworms and since then, numerous scientists have studied the effects of feeding earthworms to a variety of animals. Prayogi (2011) established that substituting 10% of the diet of quails with earthworms caused a significant increase in growth rates. A study by Orozco Almanza et al. (1988) suggested that there was no difference in the feed intake, weight gain and feed conversion of rabbits fed either 30% earthworm meal or 30% soybean meal as a protein source. Similarly, Taboga (1980) reported that earthworms used as a protein source in the diets of chickens (up to 8 weeks old) produced similar growth rates to that fed maize in a complete grower feed. The poultry industry has relied heavily on the fishmeal as a source of animal protein, but increasing costs of high-quality fish meal and declining fish stocks have resulted in renewed interest in alternative food sources with a focus on earthworm meal (Bahadori et al., 2015, 2017; Istiqomah et al., 2009; Rezaeipour et al., 2014).

Studies have also suggested that the addition of vermi-humus (organic material processed by earthworms) to earthworm meal can improve the growth and health of broiler chickens by inhibiting bacterial and fungal growth, decreasing mycotoxin levels which reduces the occurrence of intestinal diseases (Bahadori et al., 2015, 2017).

People in approximately 113 countries eat earthworms and insects (entomophagy) (Raloff, 2008). Humans in various parts of Asia, Africa and Latin America and indigenous people in Australia, New Zealand, and North America regularly consume earthworms. Although sometimes collected from the wild, most earthworms consumed by humans are farmed in a controlled environment. Recently, there has been increased interest in consuming earthworms as a sustainable alternative to "regular" meat. Compared with raising cattle and pigs, it takes less food, water and land to raise earthworms. Kangmin (2005) suggested that earthworms may be an ideal food for humans due to high protein and low lipid levels and the presence of essential amino acids. The indigenous Yekuana people of Venezuela traditionally consume two species of earthworms (motto and kuru) as the main part of their diet. Earthworms are consumed fresh, after heating in water or smoking over a fire. Paoletti et al. (2003) concluded that the nutrient contents of both earthworm species were appropriate for human sustenance. These earthworms also contain significant amounts of minerals and trace elements required by humans, particularly iron and calcium, in addition to magnesium, potassium, phosphorus and copper. The iron content of the motto and kuru earthworms is nearly 10 times greater than that of soybeans.

Earthworms grown on fruit and vegetable waste (FVW) could be a future alternative food source, whilst contributing to waste disposal efficiency. Work by Conti et al. (2019) has considered the safety aspects of this and evaluated the microbiological quality of FVW from two technological processes (freeze-drying and drying). Microbiological analyses revealed the absence of *Salmonella* spp. and *Listeria monocytogenes* in FVW, in fresh earthworms and in earthworm meal. Both freeze-drying and drying steps led to a further reduction of microbial contamination, confirming the importance of the processing methods. Earthworms may therefore represent an innovative biotechnological response to recycling of FVW, a valuable food supplement of animal proteins and a strategy to improve food sustainability.

21.2.2 In organic waste management

Litter-dwelling (epigeic) earthworms can process most organic wastes, with some modifications, discussed below. However, certain organic wastes are more favorable to earthworm growth than others. For example, of seven types of animal manure fed to *E. fetida* by Garg et al. (2005), sheep dung produced the highest earthworm growth rate and weight gain. The manure of donkeys achieved the second highest biomass gain, followed by that of buffalo, goat, cow, horse and camel. These animal manures also had differing effects on cocoon production; sheep dung yielded the most. In addition to animal manure, an extensive variety of other organic wastes have been shown to be acceptable feed material for earthworms; for example, brewery waste, potato processing residuals, paper, sewage sludge (Benitez et al., 1999; Ndegwa and Thompson, 2001), food waste (Chaudhuri et al., 2000), crop residues (Bansal and Kapoor, 2000), solid textile mill sludge (Kaushik and Garg, 2003), and spent mushroom wastes (Edwards et al., 1998).

Animal manures are produced in a variety of forms ranging from solid to almost liquid slurry. Manures are either used directly from the animal or mixed with a variety of bedding materials, such as straw, sawdust, paper and wood shavings. These wastes usually require treatment before use, as earthworms are sensitive to ammonia and inorganic salts (Edwards and Niederer, 2011). Precomposting operations can process the material to remove the ammonia and feedstock high in salts, for example, pig manure or kitchen scraps can either be pretreated in this way or washed to reduce the salt content.

Precomposting (using naturally occurring thermophilic microbes) is prevalent in commercial vermiculture operations and usually takes place for 14–21 days. The goal is to achieve sustained, elevated temperatures (to 70°C) to reduce pathogens that can be harmful to humans, animals and plants; eliminate weed seeds and reduce the heating potential of the feedstock. If organic materials are allowed to compost for longer than this period, the inherent nutrition needed by the earthworms is greatly reduced. To further diminish the heating potential of the feedstock, Edwards (2011) suggested that organic materials ought to be applied to worm beds in thin layers (2–3 cm) and that beds should be no deeper than 1 m. The additional feed should not be applied until the previous application has been consumed to avoid overheating and prevent anaerobic conditions. Epigeic earthworms need an aerobic environment and normally live in the top 10–15 cm of a worm bed and migrate upwards to consume feedstock as it is added to the system (Edwards and Niederer, 2011).

Epigeic earthworms require a moist environment, thus feedstock should be in the 80%–90% moisture range, and never lower than 60% (Edwards, 1985). The liquid should not be allowed to pool in a worm bed and overwatering may be avoided by thoroughly moistening organic materials and squeezing out excess liquid prior to addition and then periodically misting the bed with water.

The temperature within vermicomposting systems should be maintained at 15°C−25°C and should not exceed 35°C (Edwards and Niederer, 2011). During winter, a slightly thicker layer of feed can be applied (5 cm) to provide greater heat insulation, as necessary. Conversely, methods of reducing heat in a worm bed include thinner food layers, precomposting the feedstock, utilizing fans or air conditioning, and a light misting of water. In climates with temperature extremes, worm beds will likely need to be established in an enclosed, environmentally controlled structure.

Large volumes of organic residuals are being processed with earthworms using a variety of techniques, ranging from simple methods requiring significant amounts of land and labor to cost-intensive automated systems (Sherman, 2018). These techniques include 1 m high windrows; a "wedge system"; batch schemes in containers, boxes and trays which may be stacked to utilize vertical space and require less horizontal room; beds with low walls; pits; and automated, continuous-flow elevated reactors.

Outdoor windrows or beds are the most frequently used technique for vermicomposting and can be any length, but should not exceed 2.4 m in width, so the whole bed may be examined and maintained effectively. Beds are usually covered (with cloth, wood, or bamboo) to reduce the effects of direct sunlight or rain.

An automated, continuous-flow vermicomposting reactor was developed in 1981 by a team of scientists and engineers in the UK led by Clive Edwards. It consists of a 1-m deep steel vessel on raised legs with a mesh floor. Mobile gantries move horizontally across the top of the reactor, depositing a thin layer of feedstock on top of the material in the bed. About 2.5 cm of vermicompost is periodically removed through the basal mesh using a motorized, winch-driven breaker bar. The vermicompost can then be removed from underneath the reactor, either manually or on a conveyor. Organic wastes can be processed in these systems within 30−45 days.

With the exception of automated, continuous-flow reactors, all techniques are labor-intensive as they require the manual separation of earthworms from a culture medium (vermi-processed OM) with high moisture content. One method of harvesting vermicompost is to remove the top 10−15 cm of the worm bed. Most of the earthworms will be in this layer, so they may be placed on top of a new vermicomposting bed. The remaining processed vermicompost can then be removed from the original bed and any earthworms can be drawn out of this by laying a screen on top covered with food.

Another harvesting technique is sideways separation, which involves creating a new bed beside a working worm bed and applying feedstock to entice the earthworms to move. After feeding for several weeks, most of the earthworms will have moved into the new bed. A rotating trommel screen (0.6−1.3 cm woven mesh) can also be used to separate earthworms from vermicompost.

Epigeic earthworms may also have a role to play in the processing of feces produced domestically. With greater concern for the environment, dog feces are now regularly "picked up" and collected in many developed countries (Lowe et al., 2014). The presence of pathogens (*Toxocara canis*) in collected feces requires their disposal and prevents their potential use as a source of OM. However, it has been proposed that vermiprocessing of dog feces may remove pathogens providing a source of OM suitable for use as garden compost or in land restoration. In addition, as reported by Sherman (2018), human feces (humanure) can be consumed by earthworms and is already used as a feedstock. In Australia this is common in homes, businesses and wastewater treatment plants and in the USA, individuals have also added worms to composting toilets. The potential for worms to process humanure in developing countries in "tiger toilets" is enormous and shows great demand. Recent research from India and South Africa (Furlong et al., 2017) has shown that the production of appropriate worms to drive this process does not present a barrier to such vermitechnology.

21.2.3 As fishing bait

E. fetida (red wiggler) and *E. eugeniae* (African nightcrawler) have been used as bait worms since the 1940s in North America (Mason et al., 2006) and these species of earthworms, plus *P. excavatus*, are currently used as a nutritional supplement in aquaculture. The primary ingredients in cultured fish feed are wild-caught fish. However, worldwide supplies are shrinking and will shortly be unable to meet global demand. The aquaculture industry is expanding rapidly and annual production has increased from fewer than 1 million tonnes in the early 1950s to 51.7 million tonnes in 2006. The FAO of the UN estimates that aquaculture production worldwide will need to increase to 80 million tonnes by 2050 to sustain the current demand (Subasinghe, 2012).

Most cultured aquatic species have high protein requirements and supplying optimal nutrition for them comprises 40%−50% of the costs of operation and production facilities. Thus, fishmeal is the highest cost of an aquaculture operation. The raw materials for cultured fish feed are becoming increasingly scarce and higher in cost, so alternative protein sources are being investigated. The use of earthworms as a protein source for cultured aquatic species (fish and shrimp)

is gathering substantial attention worldwide. Producing earthworms locally using organic wastes is increasingly seen as an inexpensive method for supplying high-quality protein for farmed fish.

Numerous studies have demonstrated that several species of earthworms may be used as a whole or partial feed in aquaculture operations. Earthworms have been successfully fed to cultured aquatic species as live fare or in desiccated, pelleted form. For example, *E. eugeniae* and *E. fetida* have been effectively fed to carp and sturgeon (Mason et al., 2006) and *P. excavatus* fed to common carp (Pucher et al., 2012).

Earthworm meal has a protein content comparable to fishmeal (Hasanuzzaman et al., 2010; Sogbesan et al., 2007; Kostecka and Pączka, 2006) and Wing-Keong (2000) suggests that the combination of palatable and highly nutritious earthworm protein is superior to plant-based proteins, which frequently lack sufficient sulfur-based amino acids and are often unappetizing to cultured aquatic animals. Replacement of fishmeal with earthworm meal may also be beneficial to humans. Feeding with frozen earthworms (*E. fetida*) at a rate of 25%, 50%, and 75% replacement of fishmeal, resulted in a decreased lipid content of farmed Rainbow trout (Pereira and Games, 1995). Earthworms are also advocated as a replacement feed additive to fishmeal due to concerns of contaminants found in conventional sources of protein - as Hasanuzzaman et al. (2010) stated: "The quality of fishmeal is often questioned, with the presence of sand, stone, heavy metals, antibiotics, fine sawdust, poultry by-products and tannery wastes." The European Food Safety Authority also found PCBs and dioxin-like contaminants in fishmeal and recommended the replacement of fish products (Environmental Working Group, 2003; EFSA, 2005).

21.2.4 In soil restoration

Degradation of soils is a growing global problem and an issue that has arisen from both natural and anthropogenic developments and activities. Where human intervention has been central, for example, through soil removal for mineral extraction and a later desire to reinstate the soil, actions have sometimes been taken to try and recreate a living soil. Work undertaken previously often only operated in terms of the physical and chemical constituents of the soil and had not considered the structure and function of the (micro and macro-) biological components, as promoted by Bradshaw (1983). Following major landform corrections, the development of a living soil can proceed once the major components have been brought together. Constructing soils usually requires the use of subsoil and the addition of OM. This is not new, but the UK National Building Specifications (2011) dictates, that consideration should be given to earthworm augmentation or inoculation to enhance topsoil function. This long-awaited recognition takes note of research that has been undertaken over recent decades (Butt et al., 1997). Earthworms are now considered for inclusion in soil restoration due to the ecosystem services that they can provide. In other words, earthworms are able to modify ecosystem functions which are useful to humans. This occurs through the way soil-dwelling species behave in soils. As already mentioned (Table 21.1) soil-dwelling species have positive effects on (reconstituted) soils. They bring about greater aeration and water infiltration, through the provision of macropores (Shipitalo et al., 2004); but more importantly bring an improved crumb structure to the soil through ingestion of mineral and organic components and production of casting material which is highly attractive to microorganisms (Edwards and Bohlen, 1996). Mixing of soil layers and integration of the component parts permits other smaller (micro and meso) fauna to access the soil and "breathe life" into initially inert substrates, which no amount of physical manipulation could achieve.

The provision of appropriate earthworms can be brought about through mass collection (Tomlin, 1983; Steckley, 2020) or even through turf transfer (Stockdill, 1982—see Section 21.2.5), but these may be less sustainable and not as effective as earthworm culture for this purpose. With respect to this chapter, the cultivation of earthworms for soil restoration has been shown to be perfectly feasible. Butt et al. (1997), using data from laboratory-based experiments, showed that the provision of optimal conditions (Table 21.2) within a unit-based, larger-scale system, still results in the desired outcome of earthworm production. However, the innovative thinking here was to have production in earthworm inoculation units (EIUs), typically of less than 5 L, which served both as a cultivation medium and inoculation product. Starter cultures of less than 10 mature earthworms were kept within polythene bags containing sterilized soil and a suitable OM feedstock. The number of earthworms in the starter culture was a function of population density and EIU size. The EIUs were sealed, provided with air holes and maintained for a period of approximately three months at optimal conditions of temperature in an insulated, darkened polythene greenhouse. The time period was determined by the reproductive rate of the earthworm species (Table 21.2). After this cultivation stage, the intact units were transported to the proposed inoculation site (a semi-restored landfill), where they were deposited into holes of the corresponding size, and dug into the soil (Fig. 21.2). Prior to inoculation, the polythene bag was removed, and the contents were inserted as a unit, with as little disturbance to the soil matrix as possible. This was undertaken to ensure the maintenance of a

FIGURE 21.2 Two-liter EIUs transported to an organic matter enriched landfill cap postcultivation phase. The plastic envelopes, tied at the neck, are removed prior to soil-inoculation.

protective microenvironment for the earthworms, and so that cocoons deposited in the EIU remained at the same level in relation to the soil surface.

In this production system, spacing between EIUs is determined by cost and required rates of spread. At inoculation, all three life stages, adult, cocoon and hatchling earthworms are present maximizing successful population establishment. Results with monocultures of *Aporrectodea longa* (Ude) and *Allolobophora chlorotica* (Savigny) showed that this technique led to an 8 and 13 fold increase, respectively, in earthworms for inoculation during the cultivation phase and that these figures can be enhanced further by utilizing mixed-species culture (Butt et al., 1997). At the Calvert landfill site, where this work was undertaken, colonization rates from the point of inoculation were approximately 1 m/year and gave rise to typical sustainable earthworm populations after 5–10 years (Butt et al., 2004). Nevertheless, one aspect that needs further consideration here is the economics of such earthworm breeding (Butt, 2011).

21.2.5 In agro-ecosystems

In an agricultural context, the value of adding earthworms where previously absent became apparent through several serendipitous actions. For example, in an upland area of New Zealand, farmers recognized that grassland around introduced fruit trees was more productive than adjacent areas. Close examination revealed the presence of lumbricid earthworms, which were introduced with the soil around tree roots; the earthworms were shown to be responsible for the observed increase in productivity. In their absence, a thick, undisturbed mat of dead plant material had accumulated. The non-indigenous earthworms, mainly *Aporrectodea caliginosa* (Savigny), broke down the mat in the immediate area around the trees and the release of nutrients initially led to an increased grass production of over 70% (Stockdill and Cossens, 1966). So here, the actions of an endogeic species assisted plant productivity. To utilize this effect, further deliberate inoculations of similar species were performed using turf (20 cm square and 5–8 cm deep) cut from earthworm-rich pasture and laid at earthworm-deficient sites. This proved successful in New Zealand and in Tasmania (Stockdill 1982, Baker et al., 2002) with up to a threefold economic return.

Such practices although of value do not make use of earthworm culture. More recently though and building on the success of the work of Butt et al. (1997), workers in Finland addressed a field-related soil problem by breeding earthworms for inoculation. Nuutinen et al. (2006) bred field-collected *L. terrestris* and used the EIU technique to produce 7 L cultures which were introduced into the margins of an arable heavy clay soil where this species was previously absent. The rationale for this work was to increase macro porosity (through the provision of large diameter, deep burrows) for greater infiltration of water, and prevention of over-surface run-off. The inoculation took place in 1997 and the spread of earthworms has been monitored since (Nuutinen et al., 2011, 2017). The earthworms have begun to advance into the field and spread is occurring particularly in no-till plots. Over time the 1 m deep burrows are likely to link up with subsoil drainage pipes enhancing water flow through the heavy clay. Thus, the ecosystem engineers (earthworms) are interacting directly with human drainage engineers (Shipitalo et al., 2004). Here the focus is on improving agricultural systems and is similar to that of Section 21.2.4, but more applied in the context of food production.

Another potential way of breeding earthworms for agro-ecosystems (particularly in pasture) is seen more as an extensive enhancement. Here, instead of breeding earthworms in small units (larger soil-based units do not work for

reasons given by Butt, 2011)—the field itself can be utilized for earthworm culture. A suitable patch of pasture can be enriched by the application of OM. This is done to increase the breeding rate of earthworms present or species deliberately added. This thinking is not new and was proposed by Curry (1988) prior to turf cutting for enhancing endogeic earthworm numbers for inoculation. This type of earthworm culture was used in County Durham, UK by the Agricultural Development and Advisory Service to encourage the growth of earthworm populations within soil banks set aside during the process of land restoration by addition of well-rotted farmyard manure. Populations of up to 4000 earthworms m^2 were recorded under such conditions prior to inoculation. In Australia, similar measures have been employed to increase populations of *A. longa* under similar field conditions by applying OM to a pasture near Canberra, where this earthworm was introduced. The long-term goal is to use this "seed bed" as a source for further inoculations of this species in appropriate areas in the South of Australia (Curry and Baker, 1998).

In this agricultural setting, and in the context of land restoration, a variety of techniques can be used for breeding earthworms; however as suggested by Butt (2011), intensive production of soil-dwelling species is not really a viable way forward. Soil improvement through earthworm culture has a future based on two potential routes, both of which require the site of inoculation to be made as hospitable, as possible, for the chosen earthworm species (Section 21.3). Targeted breeding of specific species in small units (using the EIU technique) may be one direction forward, or a more extensive soil enrichment process may be desired for a given site that is periodically harvested for earthworm-rich turf.

21.2.6 In laboratory experimentation

Despite a recent expansion in earthworm research, predominantly in applied areas of vermicomposting and ecotoxicology, perceived difficulties associated with laboratory culture of earthworms (in particular soil-dwelling species) have led to a reliance on field-collected/commercially purchased earthworms for laboratory-based research. This practice restricts experiments to the study of juveniles or adults, often of unknown age and/or history which may compromise experimental design and validity of results (Fründ et al., 2010). The use of laboratory-reared earthworms can provide reliable and replicable experimental data and allows for the study of all stages of the earthworm life cycle under controlled conditions. While it is recognized that the extrapolation of laboratory-based results to field conditions is questionable, macro-/microcosm studies provide an opportunity to obtain fundamental ecological/biological data that is not possible in field-based studies.

Optimal culture conditions for a range of litter and soil-dwelling species have been determined (Table 21.2) and have been employed in the development of earthworm cultures and in experimental systems. This section concentrates on the control and maintenance of key abiotic and biotic factors used in the culture of soil-dwelling earthworms for laboratory experimentation. Laboratory culture of litter-dwelling species is not considered as this follows well-established practices (Section 21.2.2).

21.2.6.1 Moisture

Under laboratory conditions, evaporation of moisture from culture substrate can quickly become a limiting factor. To combat water loss, cultures are usually maintained in sealed containers with small air holes for ventilation which reduces the need for inspection to once every few weeks, without the need for re-watering. In several studies (Wever et al., 2001; Baker and Whitby, 2003) water loss was determined by weighing culture vessels on a regular basis and replenishing water as necessary. Water is usually applied to the substrate surface; however, Nuutinen and Butt (1997) cultured *L. terrestris* in PVC cylinders (6.5 cm diameter and 95 cm height) standing in a vessel filled with water to a depth of 1 cm, replacing moisture lost at the surface by capillary action. Similarly, Elvira et al. (1996) kept *L. rubellus* in 5 L cylindrical containers with a damp sponge at the base. Soil moisture conditions can also significantly influence cocoon development and a method of incubating cocoons on or between moist filter paper(s) in Petri dishes (or similar vessels) with filter paper re-hydrated as required has been widely adopted (Butt, 1991; Holmstrup et al., 1991; Garvín et al., 2002). The filter paper can also serve as a food source for hatchlings. To avoid dehydration, excess water can be added to culture vessels, submerging cocoons, which does not negatively affect cocoon development or survival of hatchlings.

21.2.6.2 Temperature

Temperature control has been used to manipulate earthworm life cycles. Holmstrup et al. (1991) recorded that *A. chlorotica* developed to maturity in 34–38 days at 20°C compared with 400 days at 5°C. Therefore, in experiments requiring large cohorts of hatchlings, low temperatures can be used to inhibit growth (enforced quiescence) until sufficient

individuals have hatched (Spurgeon and Hopkin 1999). Similarly, cocoon development can be manipulated by controlling temperature (Boström and Löfs-Holmin, 1986), as the time required for embryo development increases with decreasing temperature and may be inhibited at 3°C (Holmstrup et al., 1991) and with cocoons becoming nonviable if frozen.

Earthworms kept under constant conditions, especially at temperatures approaching the upper limits of a species tolerance can suffer from reproductive fatigue, and experience high death rates and loss in body mass compared to conspecifics under fluctuating temperatures (Uvarov, 1995). For example, Butt (1997) maintained *A. chlorotica* adults at 10°C, 15°C, and 20°C for a 12 month period during which the mean cocoon production rates were 9.9, 17.8, and 27.3 coc/ind/y, respectively, but equivalent survival rates of adults were 73%, 93%, and 15%. A trade-off is therefore exhibited between production rate and survival. Accordingly, researchers (Baker et al., 2002; Dalby et al., 1998; Fraser et al., 2003; Lowe and Butt, 1999) who have successfully cultured soil-dwelling temperate species have used a suboptimal temperature of 15°C for rearing juveniles and incubating cocoons. Earthworm cultures are usually maintained in temperature-controlled incubators or rooms (Lowe and Butt, 2002); however, other methods have also been used. Baker et al. (2002) kept cultures in a water bath while Butt et al. (1997) used an insulated polythene greenhouse with subsoil heating cables for the large-scale breeding of soil-dwelling species for use in soil restoration.

21.2.6.3 Substrate

Litter-dwelling species are usually cultured in 100% OM substrates; however, soil-dwelling species require a soil and OM mix (Butt et al., 1992; Doube et al., 1997). The reliance on field-collected earthworms in laboratory-based studies has led, with some justification, to the use of soils collected from the area of earthworm extraction as a culture medium (Daniel et al., 1996). However, field-collected soils have an inherent soil macro- and micro-fauna which, if not removed, may compromise experimental results and negatively influence earthworm survival and production. As a result, several methods of soil sterilization/macrofauna removal have been employed including sieving and hand sorting (Fraser et al., 2003), steam sterilization (Butt, 1991), microwaves (Langmaack et al., 2002) or simply air drying (Shipitalo et al., 1988). Soil sterilization is time-consuming and has led to the use of presterilized commercially available soils. Butt et al. (1994) used presterilized and sieved (<6 mm) Kettering loam with an organic content of 5% and a pH of 6.4 to culture *L. terrestris*. This soil has since been used by other researchers as a reliable substrate for the culture of temperate earthworm species (Langdon et al., 2003) and proposed as a standard medium for use in ecotoxicology (Spurgeon et al., 2004).

21.2.6.4 Feed

The preference for animal dung over other organic materials as a suitable feed for soil-dwelling earthworms in laboratory studies has been recognized since the pioneering work of Evans and Guild (1948). As a result, cow (Kostecka and Butt, 2001), sheep (Baker and Whitby 2003) and horse (Spurgeon et al., 2004) dung has been used as a feed source. Fresh/semidecomposed dung has been utilized as a food source, (Elvira et al., 1996). However, fresh dung may contain potential predators/pathogens along with a resident earthworm fauna that may compromise cultures. To achieve a consistent and reliable feed source, animal dung requires pretreatment. Langdon et al. (2003) froze fresh animal dung to sterilize and maintain its nutritional value while Lofs-Holmin (1983) recommended keeping semicomposted cattle dung in 60 L containers with air tight lids at 25°C for more than a month to kill off invertebrates and earthworm cocoons. Food particle size has also been manipulated to increase earthworm growth rates. Lowe and Butt (2003) demonstrated that the influence of food particle size was both species and life stage-specific and inversely related to size. *A. chlorotica* (adult mass 0.3−0.6 g) and *L. terrestris* (adult mass 3−5 g) were maintained in treatments of Kettering loam soil with either milled (<1 mm) or unmilled separated cattle solids (SCS). After 18 weeks, *A. chlorotica* fed milled SCS had a mean mass 185% greater than that in unmilled cultures.

21.2.6.5 Density

Laboratory-based experiments have also shown that earthworm growth, adult mass and fecundity are significantly influenced by earthworm biomass and density. Butt et al. (1994) maintained mature *L. terrestris* at two densities (4 or 8) in 2 L of soil with paper pulp applied to the surface as feed. In addition, hatchling *L. terrestris* were kept in densities of 1, 2, 4, 8, or 12 in 200 g of soil, surface fed 50 g paper pulp with 0.75 g yeast extract. Field collected mature *L. terrestris* were also kept in 300 g of soil and surface fed SCS at densities of 1, 2, 3, 4, 6, or 8 worms. Increased density had a negative effect on growth rate and final mean earthworm mass. The development of full reproductive capacity was also reduced at higher densities. In the 2 liter system, it is estimated that a mass in the range of 15−25 live g/L (3−5 adults)

may be optimum (under-reported experimental conditions) while in the smaller pots (0.3 L) fed with a superior feed, an optimal density may be 20−40 g/L suggesting that the influence of density may be modified by environmental factors.

Lofs-Holmin (1983) recommended that "small vessels should be preferred to large ones for ease of handling and sampling." It is also important that vessels are re-usable, easily stacked to maximize available space, have sealable lids to prevent excess loss of soil moisture and if cultures are maintained in the light, vessels with opaque sides should be used. These recommendations have been widely adopted (Holmstrup et al., 1991; Lowe and Butt, 2002) while novel culture vessels have also been developed to counter specific problems/requirements. Butt et al. (1997) cultured earthworms in plastic sealable envelopes (EIUs) specifically designed for inoculating earthworms in soil restoration projects (Section 21.2.4).

21.2.6.6 Species interactions

Laboratory-based research has demonstrated that soil-dwelling earthworms are capable of co-existence in mixed-species cultures (Butt, 1998) but also that species composition can reduce earthworm production (Lowe and Butt, 1999; Garvín et al., 2002). This "negative" form of interaction is species-specific and thought to result from competition for resources (feed and space). The intensity of interaction may largely be determined by the degree of niche overlap and is therefore most intense between species from the same ecological grouping (Lowe and Butt, 1999). Further research by Lowe and Butt (2002) also determined that the stage of individual earthworm development can influence both inter and intra-specific interactions These authors cultured hatchling *L. terrestris, A. longa, A. chlorotica* and *L. rubellus* in monoculture or in the presence of adults. Early growth of *L. terrestris* hatchlings was significantly greater in the presence of conspecific adults (where a high level of niche overlap would be expected); but such an advantage (possibly mediated by the availability of fragmented OM in adult middens) decreased with age. It was also noted that the behavior of anecics (*L. terrestris* and *A. longa*) changed from a subsurface (endogeic) to a deep burrowing (anecic) mode after 12 weeks ($\sim$ 1 g mass). This would suggest that in the early stages of their growth, anecic earthworms may be in direct competition for space and feed with endogeics.

21.2.7 In ecotoxicology

Earthworms possess several qualities that predispose them for use as test organisms in ecotoxicology and in particular ecological assessment of contaminated soils. They are present in most soils and are relatively sedentary, with natural immigration rates of 5 m/year (Marinissen and van den Bosch, 1992). Earthworms are both resistant and sensitive to pollutants (Cortet et al., 1999); in intimate contact with the substrate in which they live (soil); they may consume the substrate to gain nutrition and as a result, accumulate some pollutants in their tissues at higher levels than the surrounding substrate. Survival, growth and reproduction rates, in addition to the behavior of earthworms, may also be affected by pollutant type and concentration, so earthworms are considered biological sentinel species (Stűrzenbaum et al., 2009). Earthworms have played a major role in acute and chronic toxicity testing of chemicals which has led to several standardized test procedures (ISO 11268-1) (Lowe and Butt, 2007). Furthermore, the potential of earthworms as bio-indicators in field-based ecological assessment and in particular bio-accumulation studies is widely recognized (Sanchez-Hernandez, 2006).

Acute toxicity tests have relied predominantly on the use of *E. fetida* and this species has also been utilized in chronic toxicity studies. The use of this litter-dwelling earthworm is due mainly to its, short life cycle, high fecundity, ease of culture and comparable availability. However, the continued use of this species, especially in chronic toxicity studies is now questioned (Lowe and Butt, 2007). In addition, *E. fetida* is more tolerant than most earthworm species to contaminants (Lukkari et al., 2005) and therefore of limited ecological relevance. There is recognition of the need to adopt a species-specific approach in many ecotoxicological studies (Morgan and Morgan, 1998; Van Gestel and Weeks, 2004) and select test species that are representative of the site(s) under scrutiny (Svendsen et al., 2005). This has resulted in the use of a wider range of earthworm species from the three recognized ecological groupings such as *A. caliginosa* (Khalil et al., 1996); *Dendrobaena octaedra* (Rożen, 2006); *L. terrestris* (Svendsen et al., 2005). The desire for toxicity tests more representative of "field conditions" has stimulated a move toward the use of more sensitive markers such as avoidance behavior (e.g., Brami et al., 2017). Avoidance behavior may not directly influence earthworm populations, but it may lead to a reduction in beneficial ecosystem services such as reducing run-off (through burrow creation) and improving soil fertility through breakdown and incorporation of OM in agricultural systems. New, and/or, amendments to existing standardized earthworm toxicity tests to include soil-dwelling earthworms and more sensitive markers is an area that is currently under development.

While there remains a reliance on commercially purchased earthworms for use as test organisms there is a growing recognition of the benefits of using laboratory-reared earthworms (reviewed by Lowe and Butt, 2007). Earthworm origin (Lowe and Butt, 2007), age (Svendsen et al., 2005), genotype (Kautenburger, 2006) and/or preexposure (Langdon et al., 2009) can significantly influence the response to pollutants and therefore potentially invalidate results. Laboratory culture of earthworms allows the production of cohorts of known age and history (through the manipulation of environmental conditions). In addition, Spurgeon and Hopkin (1996) suggested that juvenile growth rates are a more sensitive and ecologically relevant indicator of pollution effects than adult weight change. Controlled laboratory culture allows for the production of juvenile cohorts that is not feasible via field collection due to difficulties associated with species identification of juveniles (Lowe and Butt, 2007). Earthworm culture may also increase genetic homogeneity within experimental populations which may be further enhanced by the culture of obligatory parthenogenetic species (Lowe and Butt, 2008). The latter provides the opportunity of culturing cohorts of genetically similar populations especially in species that exhibit low clonal diversity such as *Octolasion cyaneum* (Savigny) (Terhivou and Saura, 2003). This would enhance the robustness of individual studies as it allows for individuals to be maintained in isolation and provides individually monitored end points in reproduction studies. In addition, the use of parthenogenetic species would also allow for results of geographically distinct studies employing the same species to be compared more reliably. This proposal supports recommendations made by Bouché (1992) which advocated the centralized culture of specific earthworm species and strains (an "earthworm bank"—see Section 21.3) that could be distributed to laboratories to form test cultures. While this proposal has distinct advantages, protocols are required to avoid inbreeding and the production of individuals adapted to laboratory conditions.

21.3 The future for mass earthworm culture

The United Nations (2004) predicted the global human population will continue to increase and peak at 9.22 billion in 2075. This will only increase the existing demand for finite natural resources required in the production of food crops and the rearing of livestock for human consumption. The FAO of the UN (FAO, 2006) estimated that livestock grazing now uses 30% of the earth's land mass and 33% of arable land is used to produce feed for livestock. The mass culture of earthworms as a protein replacement in livestock feed and as a direct source of protein for human consumption could make a significant contribution to addressing this issue. Furthermore, as the demand for land increases, earthworms may also have a significant role to play in the restoration of degraded land and the maintenance of fertility in cultivated soils. Here, we have selected two emerging areas that may further enhance the anthropogenic benefits of earthworm culture with a particular focus on soil amelioration.

Biostimulation and Ecosystem Rehabilitation: This builds on the extensive field-based approach already described, but is wider in its thinking. Biostimulation covers a spectrum, extending from a conservation approach (with respect to existing earthworms), which encourages population development, to more active involvement in earthworm enhancement through direct inoculation into the soil or addition of OM, (vermicomposted material), which itself may have beneficial effects on soil properties (Aira and Dominguez, 2011).

The concept relies on the assertion that earthworms should be considered as part of the entire system in which they are present, rather than simply as a component. Equally, each situation to which biostimulation is applied may need to be viewed as a unique setting (with common elements to others), that lies somewhere along the given spectrum/gradient (as shown in Fig. 21.3). Where the least damage has occurred, the level of rehabilitation brought about through earthworm enhancement will be minimal and perhaps only requires a change of management practices. At the opposite end of the spectrum, direct inoculation of earthworms may be required to start the process, and this will require active land management.

Recent work (Butt, 2011; Eijsackers, 2011) clearly demonstrates that earthworms can play a significant role in the rehabilitation of soils. Sites which are earthworm-free are likely to be those which have been damaged by anthropogenic activity and therefore require soil improvement rather than simple earthworm introductions. It is also quite reasonable to introduce earthworms to fertile soils, for example, Dutch polders reclaimed from the sea, resulting in rapid earthworm population development and expansion. However, in all of these cases, the process will be rendered futile unless the necessary earthworms are available. Biostimulation, through the encouragement of population development at earthworm-rich sites, is likely to be the deciding factor.

Development of an "Earthworm Bank": The concept of storing, culturing and/or preserving biological organisms and more recently genetic resources (tissues for DNA extraction and somatic cells for culture) is reasonably well established and provides economic, conservation and long-term environmental monitoring benefits. In plants, ex situ seed storage follows well defined internationally agreed standards (Li and Pritchard, 2009). Seed banks have been

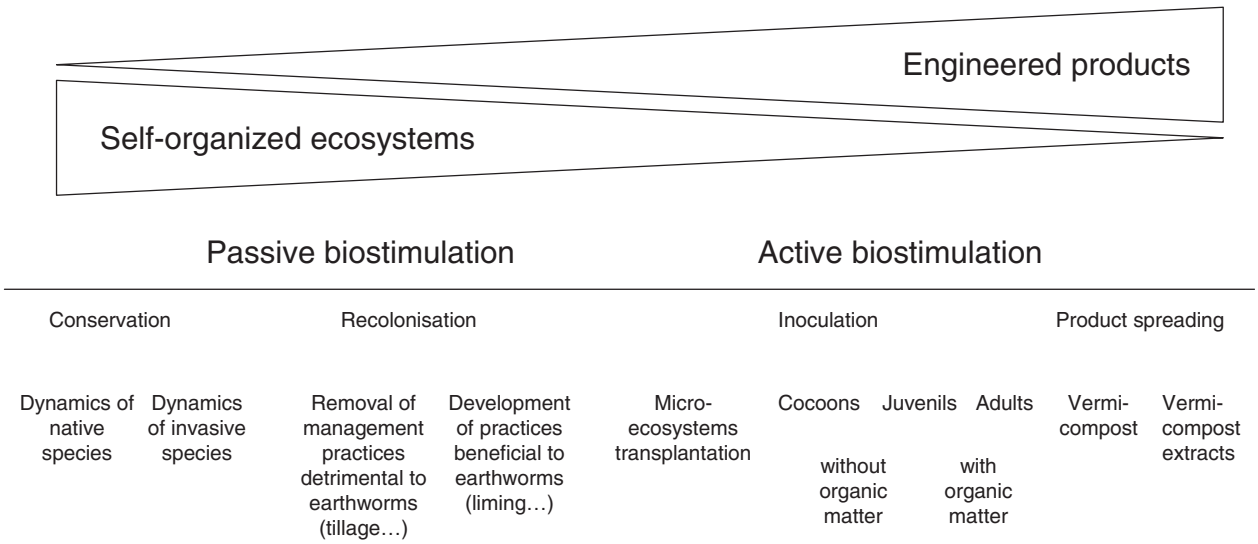

FIGURE 21.3 Use of earthworms in "biostimulation.". *Adapted from Blouin, M., Hodson, M.E., Delgado, E.A., Baker, G., Brussard, L., Butt, K.R., et al., 2013. A review of earthworm impact on soil function and ecosystem services. Eur. J. Soil. Sci. 64, 161–182.*

established to help conserve wild species (The Millenium Seed Bank at the Royal Botanical Gardens, Kew, UK) and provide genetic material for re-introduction (Cochrane et al., 2007) with the largest seed banks devoted to economically important crop species (for instance The National Center for Genetic Resources Preservation, Fort Collins, Colorado, USA) (Li and Pritchard, 2009). A number of countries have also established Environmental Specimen Banks (Germany and Denmark) to collect and store biological and abiotic samples (at low temperature $<-150^{\circ}C$) from the natural environment to allow retrospective studies monitoring pollutants, the occurrence of natural substances and genetic deviations (Poulson and Pritzl, 1993; Rüther and Bandholtz, 2009). As part of this initiative, the German Environmental Specimen Bank developed earthworm sampling and preparation techniques to assist in the monitoring of soil status. Earthworms are collected and processed by cryohomogenization and heavy metal concentrations are determined. In addition to the storage of genetic resources and samples for environmental monitoring, it has also been suggested that earthworm cultures should be established for use in applied research and commercial applications (like ecotoxicology). The development of techniques for controlled earthworm culture (particularly with respect to soil-dwelling species) and DNA sequencing, will in the future, allow for the culture of specific strains (for instance those exhibiting resistance to specific pollutants) for several commonly utilized earthworm species and potentially provide a tool for gathering important life history data on species that have not been studied. These specific strains could then be made available to researchers (as advocated by Bouché (1992)) by adapting the approach already established for the soil nematode *Caenorhabditis elegans*. This nematode is the first multicellular organism to have a fully sequenced genome; it is easily cultured and has a short life cycle. While it is recognized that there are significant differences between the culture of *C. elegans* and earthworms, the overarching principle of an earthworm bank could be similar. Studies showed that maintenance of cocoons and juveniles at low temperatures can be used to inhibit hatching and growth, respectively (see Section 21.2.6) and allow for long-term storage. In addition, the efficacy of distributing earthworms through the mail has already been established by commercial suppliers and researchers. The establishment of a centralized earthworm bank would require centralized National/International Research Council funding and active engagement of research organizations and this may not be realized or warranted until complete sequencing of earthworm species genomes is achieved.

The culture of litter-dwelling earthworms is well established, and commercial markets have been developed for the products (earthworms and compost). Fieldson (1988) questioned the economic viability of vermicomposting of organic waste. However, recently there has been a renewed interest, fueled by recognition of the resource value of waste materials (like in horticulture) and diversion of waste away from landfills. In contrast, the large-scale culture of soil-dwelling earthworms has not been commercially demonstrated. However, considering the key role that these species play in the maintenance of soil fertility and land restoration, it is only a matter of time before viable commercial operations are established. The importance of earthworms to human society has often been neglected. However, these seemingly primitive organisms may play a significant role in shaping our future existence through direct and indirect services to food provision.

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Mass Production of Beneficial Organisms

Second Edition

Invertebrates and Entomopathogens

The essential reference on producing "natural enemies" in biological control, fully revised and updated

Edited by
Juan A. Morales-Ramos
USDA-ARS National Biological Control Laboratory, Stoneville, MS, United States

M. Guadalupe Rojas
USDA-ARS National Biological Control Laboratory, Stoneville, MS, United States

David I. Shapiro-Ilan
USDA-ARS, SAA, Southeastern Fruit and Tree Nut Laboratory, Byron, GA, United States

As scientists and the public become aware of unsustainable agricultural practices and the negative impact of pesticides on human health and the environment, interest is rapidly increasing in developing biological alternatives to pest control and more sustainable sources of protein for animal feed and human consumption. In the past two decades tremendous advances have been achieved in developing technology for producing beneficial organisms such as insect predators and parasitoids, mite predators, nematodes, fungi, bacteria, and viruses. Despite this, and the globally growing research and interest in biological control and biotechnology applications, commercialization of these technologies is still in progress.

Mass Production of Beneficial Organisms: Invertebrates and Entomopathogens explores these advancements and technologies for large-scale rearing and manipulation of natural enemies while presenting ways of improving success rate and predictability of biological control procedures and demonstrating their safe and effective use. Organized into three sections, Parasitoids and Predators, Pathogens, and Invertebrates for Other Applications (particularly to produce biomass for animal feed and food), this fully revised second edition contains new information on production technology of predatory mites and hymenopteran parasitoids for biological control, application of insects in the food industry and production methods of insects for feed and food, and production of bumble bees for pollination.

This book is an essential reference for researchers working to produce "natural enemies" in biological control and integrated pest management programs. It can also be used by those researching beneficial arthropod mass production and technologies for many other uses, including rearing organisms for study and application in biotechnology and biomedical research.

Key Features

- Highlights the most advanced and current techniques for mass production of beneficial organisms and methods of evaluation and quality assessment
- Presents methods for developing artificial diets and reviews the evaluation and assurance of the quality of mass-produced arthropods
- Provides an outlook of the growing industry of insects as food and feed
- Revises methods to produce and utilize pollinator bees other than the honey bee

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