Jean-François Picimbon *Editor*

Olfactory Concepts of Insect Control - Alternative to Insecticides

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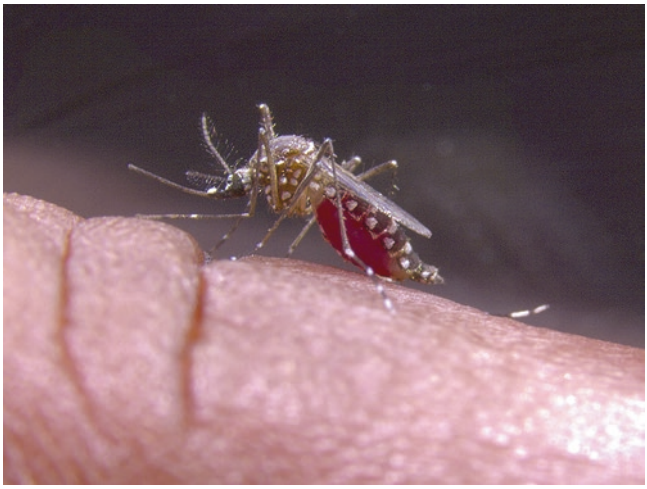

Engorged *Aedes aegypti* mosquito. (Credit to: Cédric Ramdini, Vector Control Division of the Health Regional Agency (ARS) of Guadeloupe, Saint-Martin and Saint-Barthelemy, France)

Foreword

The International Academy Partnership (IAP) has an ongoing project examining the diversity of factors that affect food security and sustainable agriculture in different countries of the world, and one obvious common theme is the impact of climate change. There are questions about how the impact of marked and often unpredictable changes in environment conditions will directly affect the growth of crops. In addition, one must consider how these impact on the distribution and dynamics of both pestiferous and beneficial insects associated with agricultural production.

At many levels the approaches taken to sustain agricultural productivity will differ between countries. However, in all instances there will be a need to control pest species while ensuring that the interventions employed have minimal effects on beneficial species, such as pollinators and biological control agents such as parasitoids and predators. While pesticides remain an important means of pest control, they have many undesirable attributes such as the development of resistance and the impact on nontarget species, including *Homo sapiens*. Therefore, it is essential that more ecologically, economically, and socially acceptable alternatives be deployed within the context of rational Integrated Pest Management (IPM) programs using to reduce the negative effects resulting from a sustained reliance on chemical insecticides.

An IPM program will integrate several different approaches to pest control, which could include the use of infochemicals, as these naturally occurring chemical cues, which modulate intra- and interspecific interactions, can be used to alter the behaviors of pests and/or their natural enemies in ways that reduce economic losses. However, an interdisciplinary understanding is required, at all levels of the system we hope to manipulate, if we wish to optimize the effective use of infochemicals. Thus, we need to have the proper identification of the infochemicals, an understanding of the molecular and physiological processes regulating the reception and integration of the chemical cues, as well as how abiotic and biotic factors influence all components of the communication systems under consideration.

The first chapters of this book address broader aspects of pest problems (both medical and agricultural) and pest management (insecticide resistance and the use of microbial insecticides) before moving into the main theme "insect olfaction."

This includes a review of both diel and seasonal periodicity in insect olfactory systems and a case study examining the potential of olfactory cues for controlling a major pest of honeybees. These are followed by reviews comparing the similarities and differences between the olfactory systems of different insect orders and pheromonogenesis, as well as those addressing the physiological, biochemical, and molecular aspects relating to the reception and integration of different infochemicals.

As evidenced by the information presented in the different chapters, there is no doubt that with the advent of new technologies, our understanding of insect olfaction has progressed significantly in recent years. However, as also noted throughout the book, there are still many questions we need to answer before the full potential of infochemicals is realized.

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Prelude

We cannot ignore it in this intriguing episode of climate change and global warming. As the year became warmer and warmer, it can become very common to hear wasps buzzing around as the whole hive built a Chinese pagoda-like nest in a corner window of the house. Meanwhile, a line of ants climbs the house walls, when vast aphid colonies drink sap, the succulent fleshy syrup aroma of leaves and stems of our secret botanical garden. In this silent cacophony, a tall and thin bright-green color morph female praying mantis in a predator threat posture hides behind a blooming flower, ready to predate in her own species, like a cannibal. She will seduce a male through eye contact, dance moves, and/or pheromonal perfume odors before devouring him as a juicy meal (Prelude Fig. 1). Then you may hear it at night; there are even crawling insect species such as cockroaches that forage for food in moist room environment in the middle of the dark. In some places, you may even hear the jaw mandibles of termites eating up wood, soil, and anything that contains cellulose. Obviously, one may share the habitat from cave to ceiling with a large number of insects (species and individuals) that soon enough will look like an outbreak that is particularly harmful.

In addition to around our homes, most of the insect species eat permanently and gluttonously all green vegetation, various fruits crops, plants, and trees. This is also a rather well-established fact. Mankind has had to fight phytophagous insects since the beginning of agriculture, horticulture, and landscape architecture following their decision to change from hunter-gathering sustained animal rearing-crop cultivation-food production to enhance their lives (Neolithic Revolution or Neolithic Demographic Transition). That was the rise of sedentary human civilization. At that time, the insect diversity was probably not as high as the repertoire in thousands of multiple biotypes and resistant strains that we discover today, but the phytophagy mode (plant feeding habit or herbivority) among insects (feeding on sterns, leaves, flowers, fruits, seeds, and roots) was surely already worrying for the green environment and our wish for development conservation. Agricultural industry was born, so was our dependence need to preserve it in order to sustain our life.

Fossils, showing all these strange creatures buried forever in clay or amber droplets, were certainly the most amazing things for men at that time. However, how

Prelude Fig. 1 Female praying mantis. (Photo taken at Gyeongju University, Gyeongju, South Korea, Credit to: Lee Klinger, Queens, New York, USA)

could they possibly foresee or even imagine any sign of warning? They probably ignored not only knowledge but also language, including any complex systems of communication in the common world. So, without knowing it, they grew plants with whom insects had established million years of relationships. We know now that some of the plant-insect duets such as *Phyllobrotica* chrysomelid beetles and *Scutellaria* flowers have developed a remarkable example of a truly intimate association (complete congruence), i.e., where "*a* cannot live without *b*, and vice-versa." How could men have ever thought that wasps cannot live without ficus and that ficus cannot live without wasps? That was only the beginning of pollination, later largely brought to our attention through bees, nectar, and most romantic sunshiny flowering plants (Prelude Fig. 2).

It is not very clear what was the food preference for insects some millions years ago, whether first insects were hematophagous (blood-sucking), phytophagous (plant-eating), and/or both. Some moth species (Geometrid fauna and the Calpinae in the Erebidae, Calyptra species of vampire moths) are known to be "ophthalmotropic" (turned toward the eye). Hosts for these parasitic Lepidoptera are not a plant leaf or a tender fruit plum but the eyes of birds and big mammals. In the savanna or in a zoo, they will seek for every exposed specimen. In a farm, they will fly over horses, cows, pigs, dogs, and eventually human and land on the eyes, waiting for the

Prelude Fig. 2 A worker honey bee of *Apis cerana* visiting *Astragalus sinicus* L. flower. (Photo taken at Kitahota, Tsukuba city, Ibaraki, Japan, Credit to: Shigeru Matsuyama, Tsukuba University, Ibaraki, Japan)

right moment to insert their harpoon-shaped proboscis in between the eyelids. These so-called "eye-frequenting" species of insects that suck the eye secretion bring cattle infectious keratoconjunctivitis, for instance. *Micropterix* but also modern moths such as pyralids chew the pollen and spores of ferns. In modern mosquito species, females suck blood, while males feed on plant or nectar, contributing to pollination just like a honeybee. In the Cambrian, much before the appearance of mankind, during the burst of so many dramatic evolutionary changes in all many various branches, the diversity of animals (and plants) was probably high enough to permit food selection but probably still limited enough to constraint food preference or choice. After the explosion of great myriads of species, there is no limitation in the insect favorite diet. That is to say, insects, in the general sense, eat everything. At a far distant Silurian time, *Peripatus*, in its cloth of velvet worm, crawled on a young forest floor among sand moisture, dead leaves, and animal carcasses. Such a mixed diet was just enough for insect kingdoms to be born.

Mankind is now a part of this world and has the responsibility somehow to cope with the fate of all on Earth, settling down, organizing homes and farms, villages and towns, cattle, food, and grain stores. Are we responsible for a second insect evolution explosion? Our incessant and unlimited activities in the recently established (in evolutionary terms) agricultural industry not to rest until high-throughput production have attracted even more and more insects that result in more and more problems. Many insects such as mosquitoes, ticks, fleas, and *tsetse* flies are known mediators of human diseases such as dengue, typhus, and many other various epidemic threat diseases. A biting tiny insect attack may have helped finish off dinosaurs through propagation of infectious lethal toxic pathogens (Oregon State University, ScienceDaily, 4 January 2008).

So, while some insects are useful to us, many are pests and condemned as guilty for a lot of crimes gifted by nature. Born with weapons (mouth in the form of scissors, wide mouth jar with tight lid, stings, various chemicals including venoms and poisonous substances, strong adhesive pads on legs, spines, horns, trunk, bristles, and hairs), many of them are now serious threats to plant, animal, and human health, when their populations reach high infestation levels. Not only ants spread germs and harvest plants not to feed but to build nests. Not only aphids, also known as plants lice, suck the life of vegetables and flowers. Not only aphids and herding ants but also varieties of beetles that are seed, leaf, or both plant eaters and the presence of a few beetles on the plant attract even more beetles. Nymphs and adult whiteflies pierce the leaf, digest the phloem, transmit viruses, induce fungus development that alter photosynthesis, change the color and edibility of plants, reduce plant growth, and cause early leaf drop, as a short list of term damage examples.

Close to flies, thrips, and an extraordinary variety of bugs among other plant suckers hidden under the surface of the leaves, moth larvae (caterpillars) feed on leaves of plants where adult females will lay eggs on stems, thereby causing severe vegetation losses not only on two different separate occasions but also on two different locations on the plant. Locusts can from gigantic ravenous swarms invade the fields of vegetation crops and destroy crops in entire countries or large parts of entire continents. The two eventualities seem to be so much more annoying than the sting of venomous wasps or bites of mosquitos. However, one should know that the result of a wasp sting varies from a single area of localized inflammation to a generalized urticarial rash, and multiple organ dysfunction syndromes can follow a single wasp sting. Common bites and allergies are not inflicted only by wasps but also by mosquitos, fleas, flies, ticks, bed bugs, lice, spiders, and many other insects and arthropods. Swelling, redness, pain, and itching are nothing compared to the emerging new forms of epidemic diseases carried by tiger mosquitos and consorts. *Nüèjí*, *huángrèbìng*, *dēnggérè*, *bānzhěnshānghán*, *Chikungunya*, and so on illustrate a disease accumulation, thanks to many types of viruses, microfilariae, and protozoa associated with many various insect strains. They all pose a deadly threat to humans.

In the insect kingdom, it seems that only the main insect pollinators, i.e., the honeybees, retain positive attitude within us. “When it is bad for the bee, it is probably bad for us” (*Bees in more troubles than ever after bad winter*, Burke and Borenstein, Associated Press Writers, March 2010). However, bees have the ability to sting similarly to wasps. How would we think of bees if they grow in uncontrollable situation? Can we predict the future expansion of bees? No, they are endangered species like many legendary birds and mammals. Bee colonies are disappearing around the world and start dying in droves, thanks to combined effects of bacteria, mites, pathogens, and neonicotinoid insecticide pesticides. While most insect species including flies, fleas, and mosquitoes developed a remarkably high insecticide resistance capacity, beneficial insects such as the honeybee seem to be much more fragile per se and much more sensitive to the high accumulation of foreign noxious toxic chemical compounds (xenobiotics) spread in the external environment, similarly to humans and any organisms living on this planet.

It has become a no-choice situation for us in order to preserve health, natural environment, biodiversity, fauna, and flora, i.e., our common heritage that is life sustaining for all. We have acquired knowledge and intelligence, although intelligence is rather difficult to define with clarity. How would we call an insect so brilliant that can circumvent and completely exhaust the whole plant defense system, find a mate or suitable crop from a kilometer distance, and migrate accordingly? How would we call a plant that calls the pinpoint right predator in response to an herbivorous attack? Nature is full of extraordinary chemicals created by us that have become pollutants one after the other. We sit on the top of the hierarchy in the tree of evolution with the unique privilege to study everything that climbs, crawls, dances, flies, grows, sings, swims, or simply lives around us. In agreement with the increasing claims and governmental concerns about ecology and environment protection, it is certainly one of our main tasks in this modern time to attempt to develop new sustainable insect pest control methods that are safe and respect the environment and public health in every form, shape, and way of implication. Finding alternatives to insecticides is prerequisite to preserve and protect human health as well as the true beneficial environmental natural fauna mediator of our culture and future.

We propose that the insect's olfactory system is not only an important driving force for their life processes but is also one of their Achilles heel. The insect antennae are covered with thousands of hairs housing olfactory neurons (or *sensilla*), which serve as micro-organs for odor detection. The surface of each *sensillum* is covered by thousands of pores through which the odorant molecules enter and access the sensory structures (olfactory receptors or ORs), prelude to odor sensing. The point is that *sensillar* pores (sites of entry of odorant molecules in the antenna) are not in direct contact with ORs.

Like human nose, mammalian muzzle, and insect antennae, peripheral olfactory organs can display all different sizes and shapes from long or short hairs to pegs and tiny scales when referring to insects. The principle basis of odor recognition is, however, the same and independent of the organ size and shape.

The external surface of ORs lays in a liquid medium, the *sensillar* lymph, which constitutes a real barrier for odorant (pheromone) molecules that are strongly hydrophobic *per nature*. In insects, odorant/pheromone molecules must be transported in the lymph in order to access the ORs. This specific task is attributed to small water-soluble transporter protein scavengers known as odor-binding proteins (or OBPs) that sieve the lymph and represent the first stage of peripheral odor recognition in insects (Vogt and Riddiford 1981; Vogt 2003, 2005).

This book (Volume 1 and Volume 2) from Springer is in continuity to *Insect Pheromone Biochemistry and Molecular Biology* (The Biosynthesis and Detection of Pheromones and Plant Volatiles; Eds. G.J. Blomquist and R.G. Vogt, Elsevier, 2003). In volume 1 of this book from Springer Nature, we will first present the current situation about expansion of mosquito vectors of pandemic infectious diseases worldwide (Chapter 1: A. Vega Rúa and B.A. Okech, *Guadeloupe Pasteur Institute*, France; *University of Florida*, Gainesville, Florida, USA). Focus will be then given to contain and control migratory locust or grasshopper swarms for which any insecticide or microbial formula can be rather difficult to apply (Chapter 2: D. Hunter;

Locust and Grasshopper Control, Canberra, Australia). We will review the evolution of the different insecticide chemical families over time, describing not only their effects on ecological environment but also the panoply of sophisticated strategies and resistance mechanisms developed by insects to counteract the panoply of drugs (Chapter 3: G. Le Goff and M. Giraudo; *CNRS UMR 7254-INRA PACA UMR 1355 - Institute Sophia Agrobiotech, University of Nice-Sophia Antipolis*, Antibes, France; Environment and Climate Change Canada, Montreal, Quebec, Canada). We will also review advances and limitations of the development of microbial biological control agents for common recurrent migratory or traditional insect pests (Chapter 4: A.P. Rooney, M.A. Jackson, C.A. Dunlap, R.W. Behle and E.J. Muturi; *USDA-Peoria*, Illinois, USA). We will then present a general overview of herbivorous pest management in a moth-crop system that cannot tolerate the use of insecticide or microbial tools; emphasis will be given in regard to the use of attractant and repellent odors as well as new gene editing techniques for sugarcane pest control and plant aroma preservation in tropical area (Chapter 5: C. Ayra-Pardo, O. Borras-Hidalgo, *Nanyang Normal University*, Nanyang, Henan Province, China; *QILU University of Technology*, Jinan, Shandong Province, China). The importance of olfactory cues in Varroa mite infection of European *Apis mellifera* honeybee colonies will further stress the unifying theme of olfaction. This will open the debate about the use of pheromone olfactory-based strategies to deal not only with insect pest control but also with the preservation of crop plant pollinators and other important beneficial insect species (Chapter 6: V. Soroker, N. K. Singh, N. Eliash and E. Plettner; *Agricultural Research Organization, the Volcani Center*, Bet Dagan, Israel; *Simon Fraser University*, Burnaby, British Columbia, Canada). Aspects of pheromone biology which will be considered include first (i) the importance of clock genes to olfactory system (Chapter 7: S. Shiga; *Osaka University*, Kansai, Honshu, Japan) and (ii) the molecular basis of sex pheromone production (pheromonogenesis) in the pheromone gland of Lepidoptera, which is the target for peptide antagonists used to disrupt mating (Chapter 8: J. Hull and A. Fonagy; *USDA-Maricopa*, Arizona, USA; *Plant Protection Institute of Hungarian Academy of Sciences*, Budapest, Hungary). And then we will extensively review the neuronal and molecular bases of the coding of sex pheromones and plant odors in insects in order to discuss about new strategies to be explored by agricultural and entomological medical industries (Volume 2).

The second part or volume of the book from Springer Nature deals with odor detection and olfactory information processing in insects. In the following, we describe: (i) the antennal sensory cell responses to single odor chemical molecule (Chapter 1: K.E. Kaissling; *Max-Planck Institute-Seewiesen*, Starnberg, Germany), (ii) similarities and differences across various insect species in coding and memorization of specific olfactory signals (Chapter 2: S.S. Singh, A. Mittal, S. Chepurwar and N. Gupta; *Indian Institute of Technology*, Kanpur, Uttar Pradesh, India), and (iii) the complexity of the insect brain to recognize specific plant odor volatiles in heliothine moth case study (Chapter 3: T. Røstelien; Faculty of Health Science and Medicine, Faculty of Social and Educational Sciences, *Norwegian University of Science and Technology*, Gjøvik, Trondheim, Norway). Then, the molecular mechanisms by which a specific pheromone or a plant odor molecule activates the olfactory sensory neurons from the insect antennae will be described, in detail.

The chapters about the molecular biology of insect olfaction focus on understanding how the insect olfactory system works at the peripheral level. Here, we will expose the central role for antennal olfactory receptors and biotransformation odorant degrading enzymes in the sense of smell and pheromone detection in insects (Chapter 4: H. Breer, J. Fleischer, P. Pregitzer and J. Krieger; *University of Hohenheim*, Stuttgart, Germany; *Martin Luther University Halle-Wittenberg*, Halle, Germany & Chapter 5: C. Steiner, T. Chertemps and M. Maïbèche; *UMR 7618 iEES-Paris, Sorbonne University, CNRS, INRA, IRD*, Paris, France). This will help develop the concept of odor transportation before activation of chemosensory/olfactory receptor cells in the insect antennae (Chapter 6: J. Zhu, I. Iovinella, F.R. Dani, P. Pelosi, and G. Wang; *Chinese Academy of Agricultural Sciences*, Beijing, China). Special emphasis will be given on a new family of odorant transporter proteins (Niemann Pick type C2 proteins) discovered in ants, revealing new key functional structures in relation with odor sensing in insects (Chapter 7: Y. Ishida; *Research Institute of Luminous Organisms in Hachijojima*, Hachijocho, Hachijo-machi, Hachijo Island, Tokyo, Japan). Concepts of transporter proteins in neurobiology of chemoreception will be further expanded to insect taste (gustation) sensory system (Chapter 8: M. Ozaki, *Kobe University*, Nada-ku Kobe, Hyōgo, Japan). Finally, we will present the molecular common interface between olfaction and immunity using the knowledge accumulated in the fruit fly *Drosophila melanogaster* (Chapter 9: E. Einhorn, and J.L. Imler; *CNRS-UPR9022, Institute of Molecular and Cellular Biology, Faculty of Life Sciences, University of Strasburg*, Strasburg, France).

We will also bring function and evolution knowledge in an analysis of the origin of moth chemosensory and olfactory binding proteins and, using insect as a model study, how evolution can possibly shape protein structure with new function via and/or mediated through RNA editing and peptide mutation (Chapter 10: J.F. Picimbon; *QILU University of Technology*, Jinan, Shandong Province, China).

The conclusion of the two-volume book in Springer Nature analyzes how insect control and/or inhibition can successfully use the knowledge relevant for olfaction and pheromone sensing. In the last part of the Springer Nature Book on olfactory concepts, we will describe how the knowledge of pheromones, plant odor volatiles, chemosensory/olfactory protein families, and structure-activity relationship can lead to the development of new efficient strategies in insect pest control, eco-drugs, specifically targeting the fundamental mechanisms of insect sensory systems while preserving human health and the environment (Chapter 11: M. Terrado, G.R. Pinnelli, J. Sanes and E. Plettner; *Simon Fraser University*, Burnaby, British Columbia, Canada, and Chapter 12: G.X. Liu, P. Arnaud, B. Offmann and J.F. Picimbon; *Shandong Academy of Agricultural Sciences - QILU University of Technology*, Jinan, Shandong Province, China; *Malaysia-France University Centre, Service for Academic and Scientific Cooperation*, French Embassy in Malaysia, Kuala Lumpur, Malaysia; *CNRS-UMR 6286 University of Nantes*, Nantes, France). Preserving human health and the natural environment is certainly one of the main common challenges of biologists, academics, and scientists, together with politicians and international world leaders, for the twenty-first century.

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Thanks to all my family and close friends for their faithful affectionate presence always.

I dedicate this book to my mother and father, "maman" and "papa", who taught me how to become but eventually also told me that life is just like a long book with many chapters.

In my life coming to Zhongguo or the Middle Kingdom, one of the chapters was written by my "sister", Véronique, who brought me two nephews, Thibault and Maxime. I wish this book published in Nature helps them, as representative of the new and future generation, that it took an incredible amount of time for a simple crust in the dust to become the earth, full of breath and life and that the tree and the

bee, the water, and the air are not here for granted and to find a place in life to achieve something as glorious as to help protect them, as we belong intrinsically together since the first human experience, is a difficult task but a necessary achievement.

We are not here to discuss who came first and how it grew or combined with what was already there to evolve and sustain in an environment full of constant changes: heat, cold, fumes, vapor gas, volcanic eruption, formation of mountains, forest soils and water flows; in this cacophony of a world to be born, a flower blossom came out with a faint suggestion of fruit to come and then the insect. The nature built all the way to the present situation now where everything is linked and lives together, and we, people or human being, are among them, i.e., all these things of life from a microscopic bacterium or algae to most curious bird or mammalian species.

We are joined in these *Olfactory Concepts of Insect Control* to discuss about the remedy or an alternative to the use of insecticides and other toxicants that threaten our natural environment. This book is an alarm call to improve from a worrying situation on many continents because of having no choice. As we focus our attention on invasive agricultural pests or mediators of human diseases, flying insects are disappearing, an evident signal that we are entering a new era that challenges for biodiversity conservation. All of us should take a common strong responsibility for rescuing actions that should be acknowledged here.

Book Abstract

We belong to the Quaternary (fourth) geological era, the "Age of Mammals," the Cenozoic era of evolution that describes 60 My, from the birth of the flower and the bee to the modern days. In a close future, men of the current time period, i.e., the twenty-first century, may soon enter a new historical era, which is made of climate change and global warming, if we did not enter it yet. The reproductive explosion of insects including highly insecticide-resistant mutant strains or biotypes in a reprogramming of the environment from many unplanned and unforeseen events leads to severe outbreaks of agriculture forest vegetation pests and diseases worldwide that cannot be matched by traditional pest control methods. The insect chemo-sense organs (olfaction and taste) are described as specific targets for pest control, alternative to xenobiotic insecticides, without affecting economic growth at the international level, but so more compatible with the protection of environment and health.

We propose to study or re-study "pheromones"; these natural chemical signals that in the insect world specifically change the behavior of another insect of the same species. While this remains a mystery in human (and most mammals), it is well known that insects have developed a most remarkable machinery for odor recognition of mates and host plants.

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About the Editor

Jean-François Picimbon Dr. Picimbon completed his doctorate in Neurosciences-Neurobiology at the University of Provence (Aix Marseille I) in 1995 after he graduated in Physiology-Neurophysiology at St Jérôme (Aix Marseille III) in France. His original work dealt with pheromone identification, regulation, and perception in moths (CNRS-INRA French Ministry of Research MRE#92114-9210 MR/MFP 907). After further behavioral, chemical, molecular, and physiological study of insect pheromones in Canada, Japan, and USA, he joined the Institute of Physiology in Hohenheim University as Alexander von Humboldt fellow (molecular basis of insect olfaction) before to be appointed "Forskarassistent" (Assistant Professor) at the Department of Ecology from Lund University in Sweden (ranked first). In 2001, his work on insect chemosensory proteins (CSPs) was honored as *"one of the top ten most talented international young scientists"* (14th NAITO Conference, Bioactive Natural Products and their Mode of Action, Kanagawa, Japan). In Lund, he led research on termite caste-specific genes, ECB moth male pheromone identification, endocrine regulation of pheromones, evolution of noctuid pheromone binding protein genes, and resolution of functional CSP structure with connection to CTBA-France (Bordeaux), INRA (Angers), School of Functional Genomics and Bioinformatics (Göteborg), National Centre for Biomolecular Research at Mazaryk University (Brno), Department of Biochemistry and Structural Biology (Lund), and various academic exchange/research programs from Belgium, Czech

Republic, France, Lithuania, and Sweden (Crafoordska Stiftelsen, Lund-Erasmus/Socrates grant programme, Swedish Research Council, and Swedish Institute in Stockholm). He moved to China in 2006 in the frame of UK-China Laboratory of Insect Biology (Nanyang, Henan Province). Following the award of Taishan Scholar and Outstanding Scientist from Abroad (2009), he is now a Distinguished Professor at the School of Bioengineering at QILU University of Technology (QLUT) and the Director of the Institute of Agricultural Microbiology in Jinan (Shandong Province, P.R. China), where he directs researches toward understanding of RNA mutations and development of new bio-natural microbial medicine and pheromone tools (eco-drug ORSA) for insect control, human health, and environment protection. He pioneered moths, chemosensory proteins, pheromone systems, and RNA editing. It is expected that the discovery of RNA editing in the silkworm moth *Bombyx mori* chemosensory gene family will pave way to key post-translational mechanisms underlying not only pheromone and olfaction in insects but also stem cell development in human. This discovery in insects may have profound impact in tissue repair and regenerative medicine, and cancer research. This research is the basis for teaching in Course 1/Ecological systems and evolution, path to biotechnology; Course 2/Sensory basis of food, wine, and juice to the path of simple enjoyment; International Course/Bioinformatics, phylogenomics and genetic tech. His thought is for teaching and education before research and innovation. His motto is, fundamental science and general knowledge should be the subject matter for applied or biotech concepts. He is also involved in developing French (Francophone) Language and Culture Education Course in support of Alliance Française in Jinan. He also helps internationalization of higher education in China through teaching "Advanced Scientific English-Science" and "Frontiers of Science": Evolution & Ecology, Animal Interactions, Pheromones, Neurobiology of Olfaction and Taste, at academic and public audience. He serves as Associate Editor of *Gene*, *Agri-Gene*, *International Journal of Bioorganic Chemistry and Molecular Biology*, *Journal of Clinical & Experimental Pathology*, *Journal of Clinical Pathology & Laboratory Medicine,*

International Journal of Proteomics & Genomics, and *SOJ Microbiology and Functional Diseases*. He is the Editor-in-Chief of *Gene & Translational Bioinformatics* (Smart Science & Technology, Houston, USA) and *International Journal of Genetic Science* (Symbiosis, Bloomington, USA).

He is matter of several biographical records including *Prabook* and "*Who is Who in Science and Bioengineering*" (insect pheromones and chemosensory proteins). He is also a recipient of Publons Peer Review Award (USA) and Insect Science Most Cited Paper Award of 2017. Besides science, he is a member of dozen international societies and governmental institutions, before all France's permanent representative to Shandong Province as ilot.SD@pekin-phedre.org in East Peninsula, North Yellow Sea, P.R. China. http://prabook.com/web/person-view.html?profileId=756595

Conference, Scientific Seminar, and Public Audience to Science

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Chapter 1
The Spread of Mosquito-Borne Diseases: A Major and Global Public Health Problem

Anubis Vega Rúa and Bernard A. Okech

Abstract Despite centuries of control efforts, the past three decades have witnessed a dramatic spread of many mosquito-borne diseases worldwide. The acceleration of urbanization, global warming, the intensification of intercontinental trade and travel, the co-evolution and adaptation between pathogens and mosquito vectors, and the development of insecticide resistance, have greatly contributed to the mosquito borne diseases worldwide. This chapter presents the current situation regarding the expansion of mosquito-borne diseases and theirs vectors worldwide, highlighting the factors that have contributed to these dramatic expansions. Furthermore, this chapter addresses the main difficulties encountered for vector control implementation using traditional approaches.

1 Introduction

Vector-borne diseases (VBD) stand as a major public health problem. They account for more than 1.5 million of deaths per year and for 17% of the estimated global burden of all infectious diseases (WHO 2014). After HIV/AIDS and tuberculosis, they are the most important cause of death worldwide (Hill et al. 2005). The VBD have in common the need of an intermediate host, usually a blood-feeding arthropod, to be transmitted between humans. Indeed, vector borne diseases are defined as infections caused by a large variety of pathogens (i.e. parasites, bacteria, viruses) that are actively transmitted to vertebrates by infected arthropods vectors such as triatomine bugs, sandflies, blackflies, ticks and mosquitoes, with mosquitoes being

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the most important vectors of human pathogens. They are able to transmit pathogens such as *Plasmodium falciparum,* which is responsible for human malaria, and more than 500 arboviruses (arthropod-borne viruses) among which more than a hundred are known to be human pathogens (Saluzzo and Dodet 1997; Gubler 2002).

Unfortunately, the available strategies for alleviating the impact of such vector-borne diseases are insufficient. Despite centuries of control efforts, the burden of vector borne diseases, particularly mosquito-borne diseases, have been constantly increasing over the last three decades (Gubler 2002; WHO 2014; Hill et al. 2005; Kilpatrick and Randolp 2012). Several conditions are required for the emergence of a mosquito borne disease: first, the pathogen (i.e. arbovirus, parasite) must be present or be imported into a region inhabited by a susceptible mosquito population. Then, the mosquito must ingest the pathogen via a blood meal taken on a viraemic or parasitemic host. In addition, the susceptible mosquito has to be "competent" to transmit the pathogen, which means that the mosquito should be able to disseminate, replicate and transmit the pathogen to a new vertebrate host during the blood feeding process (Hardy et al. 1983). Finally, the pathogen must be successfully transmitted to a new vertebrate host where the quantity of pathogen delivered is enough to trigger a new infection in an individual that in general, would be immunologically naïve to that kind of infection. Moreover, the environmental conditions (i.e. temperature, photoperiod, rainfall) are constantly modulating each one of the cited vectorial transmission steps. For instance, as the insects are ectothermic animals, the temperature conditions will importantly shape the distribution of the potential mosquito vectors (Caminade et al. 2012; Rogers et al. 2014). Furthermore, temperature modulates the vector competence (Zouache et al. 2014) and the replication efficiency of the pathogens themselves (Dohm and Turell 2001; Salazar et al. 2007), whereas rainfall plays an important role regarding the probabilities of contact between the virus and the vector. Indeed, the higher mosquito densities are generally recorded after important rain episodes (Roiz et al. 2011; WHO 2012), as they contribute to creating breeding-sites for the mosquitoes.

Which factors have contributed to the rise of the incidence and the global range of these diseases?

The global spread of mosquito-borne pathogens has undoubtedly been a consequence of the increasing global connectedness (Kilpatrick and Randolph 2012). Indeed, the globalization of the trade and travel have greatly contributed to the spread of many mosquito vector species worldwide and the pathogen importation by infected humans into new localities has been on the rise, increasing the probability of contact between the pathogens and their potential vectors. Furthermore, urbanization has enhanced probabilities of contact between the pathogens, the mosquitoes and the humans, as high densities of people are concentrated in relatively small areas that can become transmission "hot spots" with high epidemic potential. The environmental conditions that are constantly evolving in a context of climate change, have also modified the transmission dynamics, by in some cases, shortening the time lapse between the pathogen ingestion and transmission by the mosquito (Hardy et al. 1983; Vega-Rúa et al. 2015). In addition, the extensive use of pesticides in agriculture and for vector control has led to the development of insecticide

resistance, which constitutes a real problem for vector borne disease control (Marcombe et al. 2009; Bisset et al. 2011; Karunamoorthi and Sabesan 2013). Finally, co-adaptation between certain pathogens and their vectors have also contributed to some of these dramatic expansions (Schuffenecker et al. 2006; Tsetsarkin et al. 2014). In this chapter, we will review the current status of dengue, chikungunya, zika and malaria and some of their respective vectors by analyzing (i) the history of their expansions, (ii) the role of the factors cited above on these expansions, and (iii) the vector control strategies that have been implemented to fight against these emergences. As the global expansion of these diseases was preceded by the global spread of their vectors (Charrel et al. 2014), we will start by reviewing the distribution range and the multiple invasions of the mosquito vectors *Aedes aegypti* and *Aedes albopictus.*

2 The Global Spread of Mosquito Vectors

2.1 Aedes albopictus

Ae. albopictus (Skuse 1894) also known as Asian "tiger mosquito" (Smith 1956) was described for the first time in Calcutta, India. This mosquito has a tremendous medical importance as it has been involved in the transmission of several important diseases including Chikungunya and Dengue (Gratz 2004). *Ae. albopictus* was a principal vector for CHIKV in a large number of outbreaks since La Reunion epidemic in 2005 (Gratz 2004; Schuffenecker et al. 2006; Rezza et al. 2007; Grandadam et al. 2011). In addition, *Ae. albopictus* has been a DENV vector in several outbreaks in Asia (reviewed in Gratz 2004), and in countries where *Ae. aegypti* is absent (Gjenero-Margan et al. 2011). This mosquito is also suspected of maintaining the circulation of DENV in some rural areas (i.e. Bangkok) (Gratz 2004). Furthermore, vector competence experiments have shown that *Ae. albopictus* is able to experimentally transmit at least 26 other arboviruses belonging to different families such as *Flaviviridae* (genus *Flavivirus*), *Togaviridae* (genus *Alphavirus*), *Bunyaviridae* (genus *Bunyavirus* and *Phlebovirus*), *Reoviridae* (genus *Orbivirus*) and *Nodaviridae* (genus *Picornavirus*) (reviewed in Paupy et al. 2009).

Ae. albopictus is listed as one of the top 100 invasive species by the Invasive Species Specialist Group (ISSG 2009) and is considered the most invasive mosquito species in the world (Medlock et al. 2015). The ecological plasticity of *Ae. albopictus* together with the increasing human activities and intercontinental trade, have greatly contributed to the rapid global expansion of this mosquito species (Paupy et al. 2009). Indeed, *Ae. albopictus* can colonize both natural and artificial breeding sites (Paupy et al. 2009) which explains the abundance of this species in both rural and suburban sites. Studies on the biology of *Ae albopictus* have also highlighted the existence of tropical and temperate forms (Hawley et al. 1987). Unlike *Ae. aegypti*, some populations of *Ae. albopictus* in temperate regions are able to adapt

to cold temperatures and their eggs remain viable at low temperatures around 5 °C (Hawley et al. 1987). Eggs can also get in facultative diapause generally during winter that can hatch with the arrival of the first spring rains (Mori and Oda 1981; Hanson and Craig 1994; Bonizzoni et al. 2013).

In the early twentieth century, the distribution of *Ae. albopictus* was limited to the Indian subcontinent, Asia and the Indian Ocean islands (Madagascar, Mayotte, Reunion Island) (Gratz 2004). Over the past three decades, this species has spread over the five continents in temperate and tropical areas (Fig. 1.1) (reviewed in Kraemer et al. 2015). *Ae. albopictus* was detected for the first time in the Americas in 1983 (Reiter and Darsie 1984), in Europe (Albania) in 1979 (Adhami and Reiter 1998), in Africa in 1989 (reviewed in Paupy et al. 2009), and in Oceania in 1990 (Kay et al. 1990).

The intensification of trade and notably, the transportation of used tires, was responsible for the arrival of *Ae. albopictus* in the Americas (Reiter 1998). *Ae. albopictus* was first reported in the New World in 1983, in Tennessee (United States) (Reiter and Darsie 1984). Then, in 1985, the first *Ae. albopictus* breeding-site was discovered in Houston (Texas) (Sprenger and Wuithiranyagool 1986). The biological

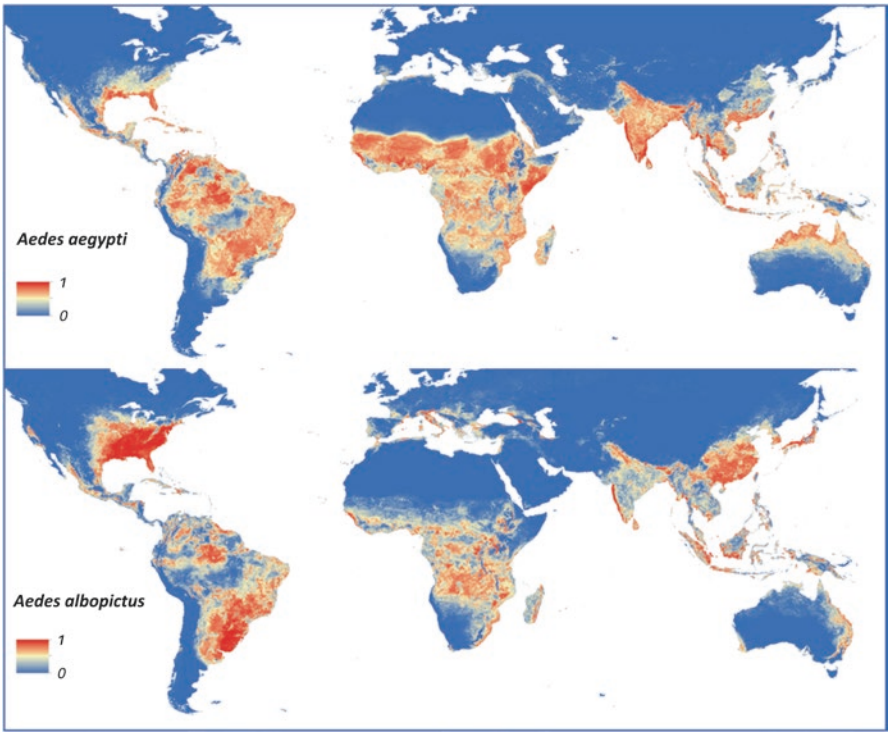

Fig. 1.1 Global predicted distribution of *Ae. aegypti* (top) and *Ae. albopictus* (bottom) in 2015. The map depicts the probability of occurrence (from 0 blue to 1 red). (Modified from Kraemer et al. 2015)

characteristics of *Ae. albopictus* populations found in the United States (i.e. cold hardiness, sensitivity to changes in photoperiod, ability to diapause), and the intensity of tires trade with Japan, suggest strongly the introduction of the species from this latter country (Hawley et al. 1987). In the coming years after its discovery in the US, *Ae. albopictus* rapidly spread to the north of Illinois and eastward to Jacksonville (Florida) taking advantage of the commercial exchanges and the road networks. Within 2 years, the species had already colonized around 113 United States counties (Urbanski et al. 2010). Then *Ae. albopictus* reached Mexico, where the first mosquitoes were found in 1988 in used tires (again) (CDC 1989). In South America, *Ae. albopictus* was first reported in Brazil in 1986 (Forattini 1986) and after, the mosquito considerably expanded its distribution range in the region. *Ae. albopictus* has been reported in Argentina (1998), Colombia (1998), Paraguay (1999), Uruguay (2003) and Venezuela (2009) (Cuéllar-jiménez et al. 2007; Navarro et al. 2013; Schweigmann et al. 2004). In Uruguay, the presence of *Ae. albopictus* has been detected near the Brazilian border, but the colonization of the country by this species is not yet confirmed (Lourenço-de-Oliveira et al. 2013). Similarly, the presence of *Ae. albopictus* in Bolivia was reported by Benedict and collaborators in 2007 (Benedict et al. 2007), but this was not subsequently confirmed (Carvalho et al. 2014). In the Caribbean, the presence of *Ae. albopictus* has been reported in Barbados (reviewed in Medlock et al. 2015), in Dominican Republic in 1993 (Pena et al. 2003), in Cuba in 1995 (Broche and Borja 1999), in the Cayman Islands in 1997 (Reiter 1998), in Trinidad in 2002 (Chadee et al. 2003), and in Haïti in 2010 (Marquetti Fernández et al. 2012). In continental central America, this mosquito has been reported in Honduras (Woodall 1995) and Guatemala (Ogata and Lopez Samayoa 1996) in 1995, in Salvador in 1996, in Panamá in 2002 (Cuéllar-jiménez et al. 2007) and Nicaragua in 2003 (Lugo et al. 2005). *In fine*, *Ae. albopictus* is now present in at least 19 countries of the Americas (Fig. 1.1).

In continental Africa, *Ae. albopictus* was first detected in South Africa (where it has been controlled) and later in Nigeria (1991), Cameroon (1999), Equatorial Guinea (2003), Gabon (2006), Central African Republic (2009), and Algeria in 2010 (Savage et al. 1992; Krueger and Hagen 2007; Paupy et al. 2009; Izri et al. 2011; reviewed in Diallo et al. 2012; Ngoagouni et al. 2015). Furthermore, the presence of the species in Brazzaville (Congo) has been confirmed during the 2011 chikungunya outbreak (Mombouli et al. 2013). Regarding Europe, *Ae. albopictus* has so far been reported in 25 countries, where this species has mainly been introduced via passive transport of eggs in used tires or lucky bamboo (Medlock et al. 2015). After its first report in Albania, the mosquito has been progressively reported: Italy in 1991 (Dalla Pozza and Majori 1992), France in 1999 (Schaffner and Karch 2000), Belgium in 2000 (Schaffner et al. 2004), Switzerland in 2003 (Wymann et al. 2008), Croatia in 2004 (Klobucar et al. 2006), Spain in 2004 (Aranda et al. 2006), and the Netherlands in 2005 (Scholte et al. 2008). Although the species was identified for the first time in France in Normandy in 1999 (Schaffner and Karch 2000), it is only since 2004 (Delaunay et al. 2009) that the species is implanted permanently in the southeast of France and began a gradual colonization of the country up to 20 departments currently (Paty et al. 2014). *Ae. albopictus* has also been established in

Greece, Malta, Monaco, Montenegro, Romania, Turkey, and has been reported but no established so far in Germany, Czech Republic and Serbia. Italy is now the most heavily *Ae. albopictus*-infested country in Europe (reviewed in Medlock et al. 2015).

2.2 Aedes aegypti

Ae. aegypti (Linnaeus 1762), the main vector of yellow fever, dengue, chikungunya, zika and many other arboviruses is thought to have an African origin (Christophers 1960; Gubler 1998; Powers and Logue 2007). In Africa, two *Ae. aegypti* forms differentiated by morphological and eco-ethological criteria are present: (i) *Ae. aegypti formosus*, dark ancestral form of various trophic preferences that lives in sylvatic and urban areas, where the larvae can breed in natural deposits (i.e. hollow rocks, tree holes, leaf axils) and in artificial containers (i.e. water storage, flower pot); and (ii) *Ae. aegypti aegypti*, lighter form known to be domestic and strictly anthropophilic that lives in urban areas, and whose larvae develop in artificial breeding-sites in and around the human habitations (reviewed in Paupy et al. 2010). This latter form is the most medically important in terms of pathogens transmission throughout the tropical and subtropical areas worldwide.

Global trade increase over the centuries contributed to worldwide spread of highly human-adapted *Ae. aegypti aegypti* (Brown et al. 2013). The general idea is that trade promoted aegypti dispersal (via eggs or even larvae/adults) all over the world. Of course, the settlement of this species occurred only where environmental conditions were favorable (i.e. tropical and subtropical area). The species was likely introduced to the Americas, the Mediterranean basin, and into Eastern and Western Africa at multiple occasions by slave trade ships between the fifteenth and eighteenth centuries (Tabachnick 1991). Then, the species spread to Asia probably during the late nineteenth century, when the first urban dengue emergences were observed in the region (Smith 1956; Tabachnick 1991; Powell and Tabachnick 2013). The transportation of humans and material during the World War has also contributed to the expansion of *Ae. aegypti*, especially in the Pacific Islands (Gubler 1998). The introduction of this species had a collateral effect on nearby all ecosystems, *Ae. aegypti* spread very rapidly in each region after all of these adoptions. Indeed, in the Americas, between the sixteenth and eighteenth centuries, the species has spread throughout the continent and already in 1950, all countries except Canada had reported the presence of *Ae. aegypti* (Tabachnick 1991; Bracco et al. 2007). *Ae. aegypti* is reported in the first half of twentieth century in Europe, in Spain, Portugal, Italy and Turkey (reviewed in Medlock et al. 2015). In Asian and Africa the species is found in most of the main urbanized cities at that time.

The increase of mosquito-borne diseases that followed the rise of infestation by *Ae. aegypti* and other medically important mosquito vectors (i.e. *Anopheles* sp) worldwide, promoted the implementation of mosquito eradication campaigns, especially as the global burden of diseases continued to increase worldwide. Indeed,

during 1950–1960, a mosquito eradication campaign based on the use of dichloro-diphenyl-trichloro-ethane insecticide (DDT) and aiming to decrease the burden of malaria as the final goal was conducted. This campaign along with the improvement of sanitation (i.e. expansion of piped water systems) and local vector control programs, lead to the eradication of *Ae. aegypti* populations from the Mediterranean basin (Gubler 1998). Also in the 1950s, the Pan American Health Organization (PAHO) undertook a campaign to eradicate yellow fever in the Americas. By the sixties, this campaign led to the decline of *Ae. aegypti* populations in almost the whole continent except Suriname, Guyana, Venezuela, some Caribbean Islands and the United States, where eradication was not achieved because local mosquitoes were particularly resistant to DDT (Soper 1963; Schatzmayr 2000). Unfortunately, due to the lack of funds and the discovery of a prophylactic mean to fight against yellow fever, the eradication campaign was stopped in 1970. Then, the explosion of intercontinental sea and air transportation have led to a re-infestation of areas where *Ae. aegypti* was previously established as well as the importation of the species into new areas (Gubler 1998; Bracco et al. 2007). Since the re-introduction of *Ae. aegypti* in Brazil in 1975 (Schatzmayr 2000), all the South American countries were re-infested: Bolivia in 1980 (Paupy et al. 2012), Peru in 1984 (Urdaneta-Marquez and Failloux 2011), the North of Argentina in 1986 (Vezzani and Carbajo 2008), Uruguay in 1997 (Salvatella Agrello 1997) and Chile in the 2000s (Bracco et al. 2007). Genetic studies based on mitochondrial and microsatellites markers have revealed the existence of multiple haplotypes and two major genetic lineages of *Ae. aegypti* in Argentina (Dueñas et al. 2009), Brazil (Bracco et al. 2007), Peru (Costa-da-Silva et al. 2005), Venezuela (Herrera et al. 2006), Bolivia (Paupy et al. 2012), Mexico and Central America (Gorrochotegui-Escalante et al. 2002), confirming the occurrence of multiple *Ae. aegypti* reintroductions in the Americas from various sources before and after the eradication program. In this sense, two possible scenarios of re-colonization of the Americas by 1970 are generally considered: (i) the spread of mosquitoes from countries where eradication has never been reached (i.e. Venezuela, Suriname, Caribbean, USA) and (ii) the dispersal of *Ae. aegypti* that survived the intensive vector control in countries where the vector was believed "eradicated" (Bracco et al. 2007). In any case, the distribution of *Ae. aegypti* in the Americas by 1995 has reached the same level as before the eradication campaign (Gubler 1998).

Today, *Ae. aegypti* occurs in tropical and subtropical areas (from 45°N to 35°S) of Africa, the Americas, Asia and Oceania (Slosek 1986; Bracco et al. 2007; Capinha et al. 2014) (Fig. 1.1). In Europe, *Ae. aegypti* is present in Madeira island since 2004 (Portugal), and in the South-East of Russia and Georgia (reviewed in Medlock et al. 2015). In Australia, the distribution of *Ae. aegypti* is confined to Queensland (Barker-Hudson et al. 1988; Muir and Kay 1998). This distribution range is explained by the intolerance of *Ae. agypti* to temperate winters. Indeed, unlike *Ae. albopictus*, *Ae. aegypti* eggs do not enter into diapause during the unfavorable season, restricting the ability for this latter species to establish in more temperate regions (Hawley et al. 1987). Nevertheless, the current global climate change can contribute to an expansion of the distribution range of this species to higher latitudes

and this can have important implications regarding *Ae. aegypti*-borne diseases transmission. Already, *Ae. aegypti* populations from temperate localities from Argentina and Uruguay have shown to transmit dengue virus as efficiently as the populations from tropical localities of the same region (Lourenço-de-Oliveira et al. 2013).

3 The Globalization of Pathogens

3.1 Dengue Virus

Dengue virus (DENV) is a positive-sense, single-stranded RNA virus that belongs to the genus *Flavivirus* (family *Flaviviridae*) and is mainly transmitted to humans by the mosquitoes *Ae. aegypti* and *Ae. albopictus* (Fig. 1.2). DENV is responsible for the highest incidence of human morbidity and mortality among all of the flaviviruses, with 50–100 million people becoming infected every year and death rates ranging between 0.03% and 1.4% (Guzmán et al. 2010). There are four dengue viruses (DENV-1, DENV-2, DENV-3, DENV-4) that are antigenically distinct but have the same epidemiology and cause similar illness in humans (Gubler 2002). Most DENV infections are asymptomatic (Duong et al. 2015) or result in mild dengue fever, which is characterized by the followings symptoms: fever, muscle, joint

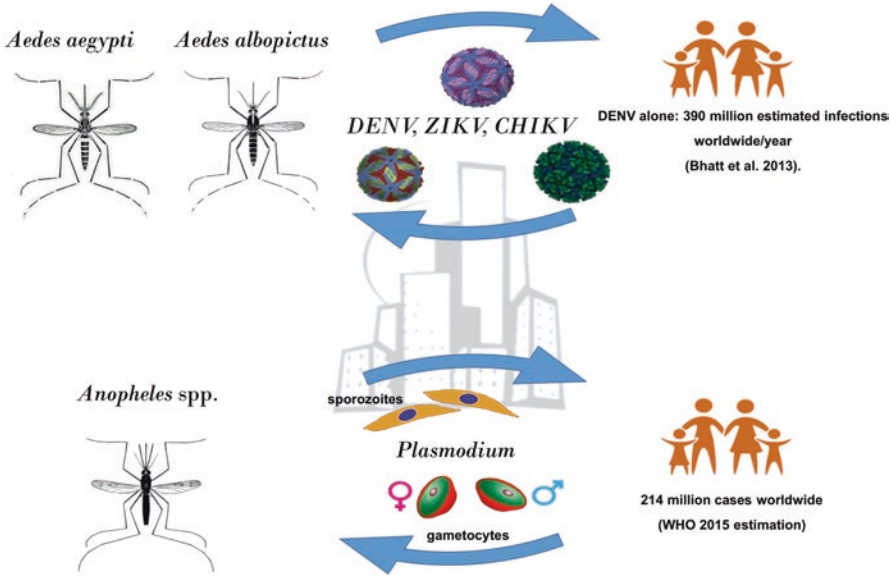

Fig. 1.2 Dengue (DENV), chikungunya (CHIKV), zika (ZIKV) and *Plasmodium* spp. vector-borne transmission in urban environments. (Source for mosquito images: Vichai Malikul/ Department of Entomology/Smithsonian Institution)

pain, and rash. However, approximately 0.5% of infections result in the most severe manifestation of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which can be fatal in 5% of cases (reviewed in Guzmán et al. 2008, 2010; Vasilakis et al. 2011). In nature, DENV is maintained in two distinct transmission cycles: (i) a sylvatic cycle, with transmission occurring between arboreal *Aedes* mosquitoes and non-human primates in Africa and South east Asia forests, and (ii) an urban cycle, with transmission occurring between domestic and peridomestic *Aedes* mosquitoes (i.e. *Ae. aegypti aegypti* and *Ae. albopictus*) and humans (Vasilakis and Weaver 2008; Vasilakis et al. 2011). The increasing urbanization of Asian and African regions where sylvatic DENV circulates contributed to the spillover of sylvatic DENV into human populations. The DENV emergence from the sylvatic cycle (ancestral) into the evolutionarily and ecologically independent urban cycles occurred independently for each serotype from hundreds to a few thousand years ago (Weaver and Reisen 2010).

DENV was spread around the world via navigation and increased trade between the eighteenth and nineteenth centuries (Gubler 1998). Dengue was first documented in the Americas, where the first outbreaks of clinical "dengue-like" syndrome were reported in 1635 in Martinique and Guadeloupe, and in 1699 in Panama (Brathwaite et al. 2012). In Asia and Africa, the first reports of major epidemics of an illness thought to possibly be dengue date from 1779 to 1780 (reviewed in Gubler 1998). By the twentieth century, epidemics caused by dengue virus were extensive and took place periodically in most of tropical areas from the world (Gubler 2002; Weaver and Reisen 2010). The ecologic disruption in the Southeast Asia and Pacific during and following World War II created ideal conditions for increased transmission of mosquito-borne diseases in that region, and it was precisely in this setting that a global pandemic of dengue began: increased epidemic transmission, co-circulation of multiple dengue virus serotypes (hyperendemicity) and the appearance of severe dengue forms DHF and DSS. The first known epidemic of DHF occurred in Manila (Philippines) in 1953–1954, and in approximately 20 years the disease spread throughout Southeast Asia and the Pacific Islands becoming a leading cause of hospitalization and death among children in the region (reviewed in Gubler 1998).

In the Americas, the development of air transport has led to the re-settlement and re-introduction of *Ae. aegypti* into new areas following the eradication program conducted during the fifties, and to a subsequent emergence of DENV outbreaks in Brazil (Schatzmayr 2000), Bolivia, Paraguay, Chile, Argentina (Vezzani and Carbajo 2008), Cuba, Puerto Rico, French Guiana and other localities (reviewed in Urdaneta-Marquez and Failloux 2011). During these epidemics, the four serotypes described for DENV invaded the Americas leading to an increased periodicity of outbreaks and the aggravation of patient clinical symptoms. In 1981, a new DENV-2 strain belonging to the South East Asia genotype (SEA) was introduced into Cuba and responsible of the first severe dengue hemorrhagic fever (DHF) outbreak and dengue shock syndrome (DSS) reported in western hemisphere causing 158 deaths (Kouri et al. 1987). Since its introduction in the Americas, the SEA genotype of DENV has been responsible for severe outbreaks involving high numbers of DHF

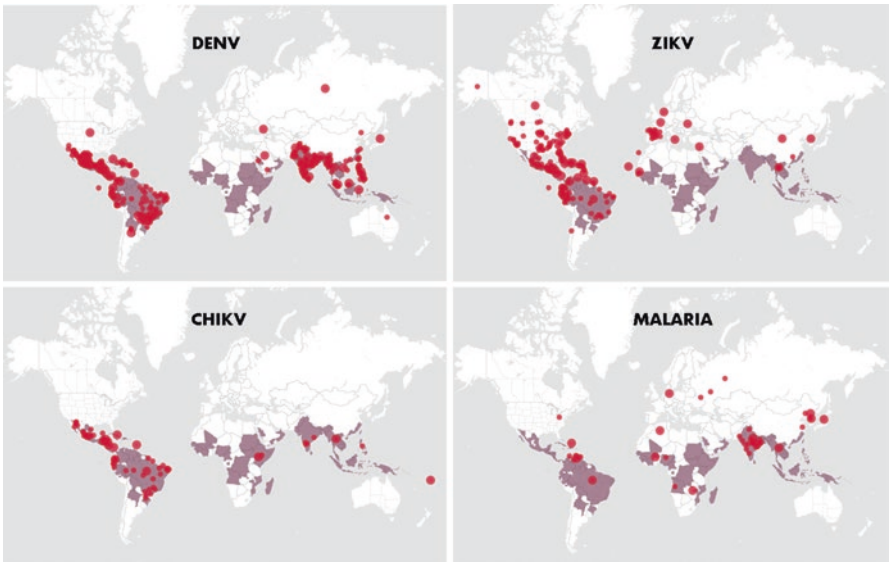

Fig. 1.3 Current global distribution and recent reports (June 2017) of dengue virus (DENV), zika virus (ZIKV), chikungunya virus (CHIKV) and malaria. (Based on Health Maps statistics (http://www.healthmap.org))

cases in several countries, and has displaced the American genotype of DENV which previously circulated in the continent (Rico-Hesse et al. 1997; Guzmán and Kouri 2008). This displacement seems associated to an enhanced replication and dissemination in *Ae. aegypti* mosquitoes of DENV-2 strains belonging to the SEA genotype in comparison with those of the American genotype (Anderson and Rico-Hesse 2006), highlithing *in senso lato* the role of arboviruses genetic variability on the vector-pathogen interactions and its potential epidemiological implications.

The current situation regarding dengue is compelling (Fig. 1.3). Before 1970, only nine countries had experienced severe dengue epidemics and today the disease is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific. One recent estimate indicates 390 million dengue infections per year (95% credible interval 284–528 million), of which 96 million (67–136 million) manifest clinically (with any severity of disease) (Bhatt et al. 2013). The Americas, South-East Asia and Western Pacific regions are the most seriously affected with more than three million cases reported in 2013. In 2015 the number of cases in these regions increased, with 2.35 million of DENV cases reported in the Americas alone of which 10,200 cases were diagnosed as severe dengue causing 1181 deaths (WHO 2016). The threat of a possible outbreak of dengue fever in Europe is more real than ever as local transmission was reported for the first time in France and Croatia in 2010 (Gjenero-Margan et al. 2011; La Ruche et al. 2010), an outbreak took place in Madeira Island (Portugal) in 2012 and since, at least 10 European countries have reported imported dengue cases

(WHO 2016). These numbers witnessed the weakness of vector control activities in the region, as well as the receptiveness of local mosquitoes to this arbovirus.

3.2 Zika Virus

Zika virus (ZIKV) is a positive-sense, single-stranded RNA *Flavirirus* (family *Flaviviridae*) of 10,794 nt (Kuno and Chang 2007) that was first isolated from a rhesus monkey in 1947 in the Zika forest near Kampala, Uganda (Dick 1952). Some reports indicates that ZIKV can occasionally be sexually transmitted between humans (Frank et al. 2016; WHO 2016), but the most important transmission mode remains the vectorial transmission via the bite of infected mosquito vectors (see Fig. 1.2). The primary vectors of ZIKV in Africa are thought to be *Aedes* mosquitoes, which is supported by viral isolations from field-caught mosquitoes such as *Aedes africanus, Aedes apicoargenteus* (McCrae and Kirya 1982), *Aedes luteocephalus* (Fagbami 1979), *Aedes furcifer*, *Aedes taylori*, and *Aedes vittatus* (Diallo et al. 2014). The human-biting mosquitoes *Ae. aegypti* and *Ae. albopictus* have been proven to be laboratory-competent vectors of ZIKV (Li et al. 2012; Wong et al. 2013; Chouin-Carneiro et al. 2016), and viral isolations were reported from both species in the wild highlighting their possible major role on the transmission of this virus (Marchette et al. 1969; Grard et al. 2014; Diallo et al. 2014). As for DENV, ~80% of ZIKV infections are asymptomatic and when symptoms occurs, they are mild including fever, itchy maculopapular rash, conjunctivitis, joints arthralgia, myalgia and headache with retro-orbital pain (reviewed in Sampathkumar and Sanchez 2016). However, recent ZIKV infections have been associated with more severe disease outcomes with neurological or auto-immune complications such as Guillain-Barre syndrome (GBS) (Oehler et al. 2014; Cao-Lormeau et al. 2016) and microcephaly (ECDC 2015; Oliveira-Melo et al. 2016).

Since its isolation, only sporadic ZIKV cases were reported in Africa and Southeast Asia until 2007, when the first large outbreak occurred in the Yap State (Micronesia), involving approximately three quarters of Yap residents (Duffy et al. 2009). Then the virus spread to French Polynesia, where it caused an outbreak from October 2013 to April 2014 involving approximately 32,000 suspected cases. During this outbreak, the virus was associated with GBS for the first time, as an unusual and unprecedented increase (20-fold more than expected) of this pathology was recorded in that period (Cao-Lormeau et al. 2016). Furthermore, an increase of new-born microcephaly cases also occurred during this ZIKV outbreak (Cauchemez et al. 2016). Subsequently, the virus spread to New Caledonia, Easter Island and the Cook Islands in 2014 (Dupont-Rouzeyrol et al. 2015; Musso et al. 2015; Tognarelli et al. 2016). In May 2015, the virus was first reported in Brazil (Campos et al. 2015), where an unprecedented outbreak began and the first association between ZIKV and microcephaly arose (ECDC 2015). Indeed, around 150 confirmed microcephaly cases were reported in Brazil between 2013 and 2014 prior to ZIKV arrival in the country, whereas from 22 October 2015 to 9 July 2016 (during the ZIKV outbreak),

the number of cases dramatically increased up to 1687 (PAHO/WHO 2016). Since then, ZIKV has explosively spread throughout the Americas and today 84 countries, territories or subnational areas have reported evidence of vector-borne Zika virus transmission worldwide (WHO 2017).

As for dengue, chikungunya, and other mosquito-borne pathogens, the intercontinental travel, as well as the high densities and susceptibility of local human and mosquito populations to ZIKV, have importantly contributed to the spread of this virus that has now reached pandemic proportions (Fig. 1.3). The organization of supranational events such as the World Cup soccer games and the World Sprint Championships, involving several thousands of visitors from different countries, may have also contributed to the transcontinental movement of ZIKV from the Pacific Island to Brazil (Weaver et al. 2016). In addition, some authors pointed out that the climatic conditions may have played a role in the recent explosive spread of Zika virus in South America, as it seems that El Niño caused exceptional climatic conditions in northeastern South America. Indeed, certain regions from Southern Brazil and Uruguay had an unusually wet winter followed by a warm summer (Paz and Semenza 2016). Finally, when ZIKV evolves it creates new molecular relationships with factors of the mosquito vector and/or the human host (Weaver et al. 2016). Thus, it is also possible than factors linked to these evolution and the interactions with the encountered mosquito vectors and vertebrate hosts, may have played an important role on ZIKV expansions since 2007. Nevertheless, knowledge about this issues are still scarce for ZIKV, and deserve therefore to be more deeply investigated in order to better understand and perhaps predict the epidemiology of this intriguing virus.

3.3 Chikungunya

Chikungunya (CHIK) is one of the major emerging diseases of the past 50 years, with a global distribution (Fig. 1.3) and the ability to rapidly move into new regions and cause epidemic disease with high attack rates. Chikungunya is an acute viral disease that has been identified in more than 103 countries in Asia, Africa, Europe – and now the Americas (Centers for Disease Control and Prevention (CDC) 2016). Phylogenetic studies suggest that the virus originated in West Africa, potentially within a zoonotic cycle that included monkeys, and/or rodents, squirrels, and birds (Chevillon et al. 2008). From this source, it appears to have spread to Central, Southern, and East Africa (ECSA clade), and from there moved to Asia (Asian clade) and to the Indian Ocean Islands. In contrast to the ecologic picture seen in West Africa, illness in Asia and the Indian Ocean region has generally appeared in the setting of large, sporadic outbreaks. In 2005–2006, approximately 272,000 people were infected on the Islands of Reunion and Mauritius, followed, in the same year by an outbreak in India where close to 1.5 million people were infected (WHO 2007). In 2007, the first European CHIK outbreak took place in Italy (Rezza et al. 2007), and subsequently, autochthonous cases have been reported in France in 2010

and 2014 (Grandadam et al. 2011; Delisle et al. 2015). In December 2013, CHIK arrives to the Americas and rapidly spread throughout the continent reaching more than 46 countries and/or territories (http://www.cdc.gov/chikungunya/geo/index.html; Fig. 1.3). Today, estimates indicate that close to 39% of the global population live in areas with a high risk of CHIK transmission and between 33,000 and 93,000 clinical cases occur *per annum* with case fatality rates between 0.1% and 2.8% (Labeaud et al. 2011). It is also believed that the reported cases of CHIK represent just a fraction of the cases which actually occur, due both to underreporting and a relatively high rate of asymptomatic infection (Nakkhara et al. 2013; Appassakij et al. 2013). Although the fatality rates associated with CHIK infections are low, morbidity is high and the DALY's associated with CHIKV infections make it of significant concern for public health agencies.

Illness is characterized by acute onset of high fever, with potentially disabling polyarthralgias. In most instances symptoms of the disease resolve spontaneously within 7–10 days. However, depending on strain and host population, joint symptoms can persist for months to years. In a case series from Marseille (patients returning from the Indian Ocean region), 46% of patients reported persistence of joint symptoms for at least 6 months after acute illness (Simon et al. 2007); in a study from South Africa, 12% of patients reported arthralgias 3 years after acute illness (Brighton et al. 1983). There is also increasing recognition of more serious complications and deaths, particularly among children, including encephalitis, febrile seizures, and acute flaccid paralysis. It is unclear whether these more severe symptoms are the result of better reporting, in a setting where diagnostic tests are available; are a function of increasing virulence of the strain; and/or reflect changing host immunologic responses. The strain that has reached the Caribbean in December of 2013 belongs to the Asian clade (Lanciotti and Valadere 2014; Leparc-Goffart et al. 2014).

The transmission of CHIK may be urban or sylvatic, especially in Africa where several arboreal *Aedes* species are involved in a sylvatic transmission cycle (Diallo et al. 1999). In the urban cycle (see Fig. 1.2), transmission is often sustained by the peri-domestic *Ae. aegypti* and *Ae. albopictus* (Reiter et al. 2006), although other local *Aedes* mosquito species may also play a role in CHIKV transmission (Diallo et al. 1999). However, in new areas to which CHIK spreads there is a need for detailed investigations to confirm the role of other local mosquito species in CHIK transmission dynamics. Both mosquito species are known to co-occur in rural and urban environments (Juliano et al. 2004) although *Ae. aegypti* predominates in urban settings and *Ae. albopictus* in rural settings (Lourenco-de-Oliveira et al. 2004). There is also evidence that *Ae. albopictus* is predominant in many environments where they co-occur (Braks et al. 2004). The efficiency of viral transmission by either vector seems to be influenced by single point mutations in the viral envelope protein and it was shown in the Indian Ocean outbreak that a mutation in the viral envelope protein (A226V mutation) led to increased fitness of CHIKV in *Ae. albopictus* (Tsetsarkin et al. 2007; Vazeille et al. 2007) that enabled its rapid expansion. Other investigators have found that *Ae. aegypti* has a better transmission efficiency for the original CHIK viral strain (African strain) than the Asian genotype

(Vega-Rúa et al. 2014). As previously stated, the extensive distribution of *Ae. aegypti* and *Ae. albopictus* in many parts of the world has increased the risk associated with arbovirus transmission (Gratz 2004).

3.4 Malaria

Malaria, a mosquito-borne disease caused by a parasitic protozoan in the genus *Plasmodium*, continues to present a global public health threat with a presence in close to 100 countries around the world (Fig. 1.3). There are more than 120 species of *Plasmodium*, but only five are infectious to humans. Four of these species cause widespread infections in humans including *P. falciparum* that is responsible for the most severe malaria, and *P. vivax* is the second important cause of malaria and is most prevalent in Asia, the Americas and the Pacific and *P. ovale* and *P. malariae*, both of which occur in Africa, particularly in Central and Western Africa. The little known *Plasmodium knowlesi*, is a zoonotic malaria infection commonly found in Gorillas and chimps, but also has the ability to infect humans (CDC 2015).

The public health importance of malaria can be surmised by the World Health Organization that estimates that 3.3 billion people are at risk of malaria infection world-wide (30% of which are at high risk) (WHO 2015b). Estimates available indicate that more than 200 million malaria infections occurred globally with approximately half a million deaths, most of which were in Africa. Of these deaths, most were children under 5 years old (WHO 2015a). In the Americas with a combined population of 895 million people, 61% of the population is at a high risk of malaria infection (WHO 2015b). More than three million cases of malaria occur in the Americas, of which 39% were reportedly due to *P. falciparum* (Hay et al. 2010).

Anopheles mosquitoes are the primary vectors of malaria (see Fig. 1.2) with at least 40 species of Anopheles mosquitoes having been identified to be capable of transmitting malaria. In Africa, the predominant mosquito vector is *Anopheles gambiae* and *Anopheles funestus*. The ranges of these mosquito vectors extend from South America to the Sahel in western and northern Africa. In the Americas and Asia, several species complexes are involved the transmission of malaria. The distribution range of *Anopheles albimanus* is expected to increase in the face of land cover changes in South America (Alimi et al. 2015). In Asia, *Anopheles stephensi* plays the critical role in malaria transmission. The distribution of malaria vectors is influenced in part by the availability of breeding sites, host blood mean sources, which in turn affects malaria transmission. The transmission dynamics of malaria varies depending on vector behavior. In most areas of sub-Saharan Africa, both indoor and outdoor biting malaria vectors sustain transmission. The fact that transmission occurs both indoors and outdoors has complicated the IRS program for malaria control, leading to the inclusion of outdoor spraying for malaria control. In many malaria endemic areas of the world, great strides have been made to reduce malaria incidence, however measures that have been put in place must be sustained

because historical evidence has shown that malaria cases can resurge even in places where it was previously brought under control. Some of the factors that are responsible for the resurgence of malaria include resistance to insecticides and to antimalarial medications. The classic example of where malaria was controlled and later resurged is Sri Lanka where in the 1970s malaria levels were brought to down to less than 1%, and due to the lack of sustainable control measures coupled with insecticide resistance, there was a resurgence of malaria (Dutt et al. 2010). In Zanzibar, the use of effective medications and widespread ownership of ITN's led to significant reductions of malaria. The continued high coverage of malaria interventions should be continued to sustain the gains. However, as has been observed in many countries, resurgences in malaria has been most caused by a lack of resources (Cohen et al. 2012).

In recent years, increasing temperatures have resulted in the malaria vectors shifting from their traditional locations to invade new zones (Ngarakana-Gwasira et al. 2016). In fact, climate change is considered the most important factor that might influence malaria transmission in high elevation areas where malaria transmission could never be sustained. In other instances, the changes in land use patterns, including agricultural developments, deforestation and large scale infrastructural developments such as the development of dams and hydroelectric power plants have result in the creation of favorable habitats for malaria mosquitoes breeding, which in turn has led to an increase in malaria transmission (Tompkins and Caporaso 2016). In addition, such developments attract human settlements from people moving in search of jobs and livelihoods.

4 Mosquito Vector Control Strategies

Over the first half of the twentieth century when organized mosquito control was started, there was heavy reliance on chemical insecticides (Simmons and Upholt 1951). One of the most controversial insecticides ever used in mosquito control was the chemical known as dichloro-diphenyl-trichloroethane or simply DDT. It was an environmentally safe chemical because of its persistence in the environment and was therefore taken up in the food chain. Yet it was able to reduce the burden of major vector borne diseases in large parts of the world. DDT was particularly effective in reducing the burden of malaria in the world during the global push by the WHO malaria elimination program of the 1960s (Najera et al. 2011). The side effect associated with DDT use (environmental side effects and persistence), sparked (spurred) the search for insecticides that were environmentally benign. Many classes of insecticides have been developed since that are very effective in controlling mosquitoes but with less environmental impact on non-target species and in contaminating soil and water. It is also very important to minimize the development of insecticide resistance to these chemicals. Therefore, strategies have been implemented to supplement, and in some cases, totally replace the use of insecticides that have been rendered ineffective due to resistance. The WHO panel of experts on

Fig. 1.4 Surveillance and mechanical destruction of *Ae. aegypti* breeding sites in Saint Martin Island by workers from the Regional Agency of Health and the Institut Pasteur of Guadeloupe. (Photo: Laboratory of Medical Entomology-Institut Pasteur of Guadeloupe)

mosquito control has recommended the use of Integrated vector management (IVM), which essentially provides guiding principles for the control of mosquito vectors with minimal impact to the environment (WHO 2004). Integrated Vector Management is an old concept that was first developed and applied by William Crawford Gorgas to eliminate Yellow Fever from Havana in the summer of 1901, and to control malaria transmission during construction of the Panama Canal. Its principal components are complementary keeping infected mosquitoes away from people with bed nets and spraying residual insecticides to kill infected mosquitoes, as well as environmental modification and larviciding that would reduce the sources of mosquitoes by removing stagnant water and other breeding sites (Fig. 1.4).

4.1 Insecticides

Mosquito control in the pre-DDT era relied heavily on very toxic chemical products. In the US, as in other parts of the world, there was extensive use of Paris green and petroleum by-products (Simmons and Upholt 1951). With the discovery of DDT as pesticide and its widespread application, it enabled the eradication of many vector borne diseases, and most notably malaria (Simmons and Upholt 1951). After its introduction in 1946, the detrimental effects of DDT were made public leading to a re-evaluation of its use. The United States Environmental Protection Agency has classified DDT and its metabolites, DDE and DDD, as persistent chemicals in the environment because they do not biodegrade and can take nearly 15 years before they are no longer present (EPA Division Health and Ecological Criteria 2008). There is also the risk of bioaccumulation in the food chain, resulting in high exposure levels for humans leading to damage to the liver, nervous system, and reproductive system (EPA Division Health and Ecological Criteria 2008). In an effort to protect people and the environment, the Stockholm Convention on Persistent Organic Pollutants restricted the use of DDT in 1996, when they stated that as long as "locally safe, effective and affordable alternatives are available" then DDT should not be used. However, in 2004, DDT was restricted to malaria control in areas where

local, safe and effective and affordable alternatives were unavailable. With many countries banning the use of DDT, safer chemical alternatives that were benign to the environment but equally effective and mosquito control were developed. Several insecticide classes have been developed including chemical such as pyrethroids, carbamates, organophosphates, insect growth regulators (IGR's) and biological such as *Bacillus thuringiensis* (var *israelensis*), RNAi, and other that are biological classes of insecticides. Many of these insecticides have been used very effectively in the control of mosquito vectors and vector borne disease, but their use has led to the appearance of insecticide resistance in many mosquito vector populations (Karunamoorthi and Sabesan 2013), which highlight the need to review the current vector control strategies regarding insecticides.

4.2 Environmental Management

Environmental management for mosquito control according to the World Health Organization (WHO) is defined as the planning, organization, implementation and monitoring of activities that modify and/or manipulate environmental factors or their interaction with man with a view to preventing or minimizing insect vector propagation and reducing man-vector-pathogen contact (WHO 2004). It entails either or both of the following: (a) environmental modification – permanent infrastructural changes of a capital-intensive nature, (b) environmental manipulation - recurrent actions aimed at achieving unfavorable conditions for vector breeding. In the pre-DDT era, much of the major vector control activities consisted of both environmental modification and manipulation and changes to human habitations and behavior. Drainage of potential mosquitoes breeding sites (wetlands, marshes) was undertaken largely by digging of canals. However, since wetlands are recognized as ecosystems themselves that are needed to protect the biodiversity, changes have been made in how wetland ecosystems are managed for vector borne disease control. Proper management and implementation of major environmental manipulation projects is needed for protecting the environment while at the same time ridding the mosquito problem.

4.3 Genetic Tools

The idea of genetic control of vector borne diseases was first used effectively to control the sleeping sickness in Tanzania when sterile hybrids were created by the interspecific mating of *Glossina swynnertoni* a vector of Trypanosomiasis with a non-vector *Glossina morstians centralis* (Vanderplank 1947; Vanderplank 1948). In these crosses, males were sterile and female hybrids were partially sterile with the idea that gradually, the entire population would be replaced by these sterile and non-vector species. This set the stage for the use of genetic strategies for vector borne

disease control. Several methods have been tried that utilize genetic mutations or modifications to reduce mosquito reproduction including sterile insect technique, the release of Insects with Dominant Lethality (RIDL) (Thomas et al. 2000) and the use of the endosymbiotic bacteria *Wolbachia* (Slatko et al. 2014; Caragata et al. 2016) among others. The control of mosquito populations through population replacement with mosquitoes that are either refractory to the pathogens, or that do not produce viable offspring might help in the control of mosquito borne diseases (Wood 2005). The use of RIDL technique developed and tested by Oxitec© has been shown to induce premature death in progeny and to have very good results in mosquito population suppression (Atkinson et al. 2007). However, the use of genetic modified mosquitoes is a highly controversial issue that is hampered by different legislations depending on the region. In addition, as the RIDL approach is not self-sustainable (i.e. need to continuously produce and release RIDL mosquitoes), its application seem more suitable in small island rather than in large continental areas. *Wolbachia*, an endosymbiote rickettsia-like bacteria that naturally infects cytoplasmic vacuoles of insects including mosquitoes (Beard et al. 1998), has the ability to alter their reproduction, and has also shown great promise in the fight against arboviral diseases. The *Wolbachia* infection causes cytoplasmic incompatibility and therefore mosquitoes that are incompatible will not have viable offspring. For a detailed review of the *Wolbachia* and mechanisms of CI and how it has been used as a paratransgenesis tool, refer to (Beard et al. 1998). Many field trials with *Wolbachia*-infected mosquitoes for the control of dengue have been tried out and the evidence is strong that *Wolbachia* infected mosquito reduce the transmission potential of Dengue (Ye et al. 2015; Lambrechts et al. 2015). In addition, evidence from other studies indicate that *Wolbachia*-infected mosquitoes reduces or blocks Chikungunya and Zika transmission (Aliota et al. 2016a, b). However, other studies have shown that *Wolbachia* might enhance infection with WNV (Dodson et al. 2014) in *Wolbachia*-infected *Culex tarsalis*. Furthermore, as *Wolbachia*-infected mosquitoes are laboratory strains that are probably susceptible to insecticides, the success of this approach requires the suspension of insecticide treatments in concerned localities. Otherwise, *Wolbachia*-infected mosquitoes will be all killed after the first insecticide treatment and only the resistant wild-type local mosquitoes will survive.

4.4 Future of Vector Control

As vector borne disease spread across the globe and into new areas, it will be necessary to apply a multiplicity of control tools available but also to develop new tools of greater specificity and respectful of the environment. The idea of integrating several vector control tools is not a new one but the concept of rational decision-making process for optimal use of resources for vector control that forms the core of IVM is key to the sustainability of vector management approaches (WHO 2004). In the past, it has been difficult to implement the IVM strategy but the refining of the

different components and the discovery of new tools should allow that many vector control programs adopt IVM. These components include collaboration, integrated approach, evidence based decision making, capacity building and advocacy, social mobilization and legislation.

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Chapter 2
Locusts in the World

David Hunter

Abstract Locusts (Orthoptera: Acrididae) form dense bands and swarms that can cause substantial damage to pastures and crops. And a feature that makes locusts particularly devastating is the ability of migrating swarms to appear without warning in large numbers in previously uninfested areas, overwhelming local crop protection programs. To reduce damage, many governments conduct locust control programs: they aim to begin treating the locusts *before* they reach crops to try to reduce the size of swarm invasions of cropping areas. Locusts alternate between periods of low numbers (recessions) and very high numbers (plagues). When in low numbers, locusts are quite dispersed, but favorable conditions allow populations to increase and the dispersed locusts undergo a behavioral change where they come together to form bands and swarms. Most locust management programs rely on regular monitoring of locust populations and when bands or swarms are detected, treatment programs begin. But these early intervention programs have the greatest chance of success if they are combined with a reasonably good understanding of the critical factors that lead to population upsurge and of where locusts are more likely to be. When such factors occur, then extra resources need to be made available for survey and treatment. It takes several generations of successful breeding for initial localised outbreaks to reach plague proportions: and the aim is to rapidly find and treat as many of the locusts as possible in each of the generations of increase as part of a strategy of preventive control (*la lutte préventive* or *la lucha preventiva).* Such treatments can limit the rate of increase in each generation, reducing the consequent damage when the locusts reach crops. Treatments have relied on the use of various chemical pesticides, and the use of chemical pesticides will continue. But increasing constraints on the use of chemical pesticides mean that alternatives need to be investigated as part of treatment programs to ensure locusts are treated whenever and wherever they are found.

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1 The Locust Problem

What the crawling locust left, the swarming locust has eaten;
What the swarming locust left, the hopping locust has eaten;
What the hopping locust has left, the stripping locust has eaten.
Joel 1:4

When locust populations are high, they form dense bands of marching nymphs (Fig. 2.1). And when the locusts reach the winged adult stage, they can form high density swarms of flying adults (Fig. 2.2). At such high densities, they can cause substantial damage to agriculture, which has led to intense control efforts to limit such damage. In periods between such high-density outbreaks, locust populations are often quite low and are hardly noticed, being scattered in their environment much like their fellow grasshoppers of the family Acrididae.

Like grasshoppers, locusts have a three-stage life cycle: ***eggs*** laid in the soil that hatch out as ***nymphs*** that go through five (up to seven in some locust species) moults as they grow to reach the winged ***adult*** stage. When conditions are particularly favourable, populations of both locusts and grasshoppers increase but the major difference is that when in high numbers, locusts crowd together attracted by visual and olfactory cues (Simpson et al. 1999). With many locust species, adults crowd together to lay in dense eggbeds where groups of adults lay their ***eggs*** by burrowing into the soil with their abdomens (Fig. 2.3a, b). The eggs are laid in eggpods, each of which contains 50–100 or so eggs, depending on the species, and when adults crowd together to lay, the resulting eggbeds have thousands of eggs per square meter. After 2–3 weeks in summer, the huge numbers of ***nymphs*** hatch out and

Fig. 2.1 A section of a dense band of the Moroccan locust *Dociostaurus maroccanus* (Thunberg) in Uzbekistan, April 2011. (Photo by David Hunter)

Fig. 2.2 A flying swarm of the Central American Locust, *Schistocerca piceifrons piceifrons* in the Yucatán, Mexico 2009. (Photo by Jim Conrad)

Fig. 2.3 Adults laying and nymphs. (**a**) Adults of *Schistocerca cancellata* in Argentina grouping together to lay. (**b**) Closeup of a laying adult with its abdomen in the ground. (**c**) A late instar *S. cancellata* nymph showing bright colors of gregarious phase. (Photos by Maria Laura de Wysiecki). (**d**) Damage caused by a band of *Locusta migratoria* invading a young sorghum crop in Australia. (Photo by David Hunter)

group together and soon form marching bands that range from moderate (~30/m^2) to very high (1000/m^2) density (see Fig. 2.1).

With many locust species, the nymphs in these bands look quite different than the isolated nymphs seen at lower densities, often being of quite striking colours (Fig. 2.3c). And when nymphs at the densities seen in Fig. 2.1 invade crops, they can cause substantial damage, virtually stripping many the plants to ground level (Fig. 2.3d). When such bands of nymphs reach the adult stage, they can form dense swarms of adults that cause a similar high level of damage.

The differences between the cryptic nymphs seen at low densities and the colourful high-density nymphs are so dramatic that they were once thought to be different species. But Uvarov (1921) demonstrated for the migratory locust that there were two forms of the same species. At low densities, migratory locusts are in the *isolated phase* where they have cryptic coloring and behave as individuals well hidden from predators. But when populations are high, the locusts undergo a *phase change* where they change to the *gregarious phase* characterized by dramatic differences both in behaviour (in forming bands) and in having striking body colors. While there is debate about the advantages of being so obvious, it is clear that it is impossible for locusts in high numbers to remain hidden, so there is no longer any advantage to having the cryptic coloration and behaviour seen when populations are low. Locusts remain in the nymphal stage for 5–8 weeks depending on the temperature and locust species, and then the locusts reach the winged ***adult*** stage, where the locusts can form high density swarms at densities of 50–100 individuals/m^2 (see Fig. 2.2). These swarms can often fly hundreds of kilometers and this ability of migrating swarms to suddenly appear in previously uninfested areas is a characteristic that makes locusts such devastating pests.

However, while some locusts have all of the features "characteristic" of locusts (dense laying, bands, swarms, migration, phase change), others do not. This is not surprising given that the ability to form bands and swarms has evolved *independently* a number of times in the various subfamilies of the Family Acrididae (Song 2005, 2011). And even the factors that *lead* to gregarization differ between species: in the desert locust, *Schistocerca gregaria* (Forskål), gregarization is evoked by touching of the femur of the hind legs (Simpson et al. 1999, 2001), which would mean that gregarization is evoked by locusts brushing against each other when numbers are high. With the Australian Plague Locust *Chortoicetes terminifera* (Walker), gregarization is triggered by tactile stimulation of the antennae (Cullen et al. 2010). In addition to these tactile responses, there are effects of odours on gregarious behaviours such as group oviposition but the varying results observed among the locusts studied suggest a variety of mechanisms in the different locust species (Ferenz and Seidelmann 2003). Species that have all of the features characteristic of locusts, such as the Migratory Locust *Locusta migratoria* (Linnaeus) have been designated by some authors as true "model" locusts, in contrast to "nonmodel" locusts that exhibit only some of the factors (Song 2011). However, from personal observations, Australian species are seen to form a continuum from locusts that have all of the characteristics typical of locusts through to grasshoppers that have none. The Australian Migratory Locust has all of the characteristic features (dense

laying, bands, swarms, migration, phase change) while the Australian Plague Locust has dense laying, bands, swarms and long distance migration but no clear physical phase change. The Spur Throated Locust, *Austracris guttulosa* (Walker) rarely forms bands but has very dense swarms that migrate, while the Small Plague Grasshopper, *Austroicetes cruciata* (Saussure) can have small bands, phase change and low density swarms that move only a few tens of kilometres. There are some *Austroicetes* species that have swarmlets that fly short distances and then there are many grasshoppers that have adults that fly only as individuals.

2 The Aim of Locust Management Programs

Locusts require particularly intense management programs. Whether locusts are "model" and "nonmodel" types is not important: what is important is whether they can form bands or swarms as it is locusts in bands or swarms that can cause substantial damage in a very short period. Locusts that have had major outbreaks or plagues in the past half-century are found in most of the warmer parts of the world from Africa to Asia to Australia to North America (Mexico and south) to South America (Fig. 2.4). There was even a locust problem in the USA: the Rocky Mountain Locust, *Melanoplus spretus* Walsh, formed huge swarms in the late 1800's but it became extinct, likely because of land use changes (Lockwood 2004).

Clearly locusts are a widespread problem and a feature that makes them particularly devastating is the ability of migrating swarms to suddenly appear in large numbers in previously uninfested areas: when invading swarms are of high density, they can virtually destroy a crop within a day or so, before there can be any effective crop protection treatments by landholders. Consequently, it is well recognized that locust treatment programs must be more than just crop protection and most programs

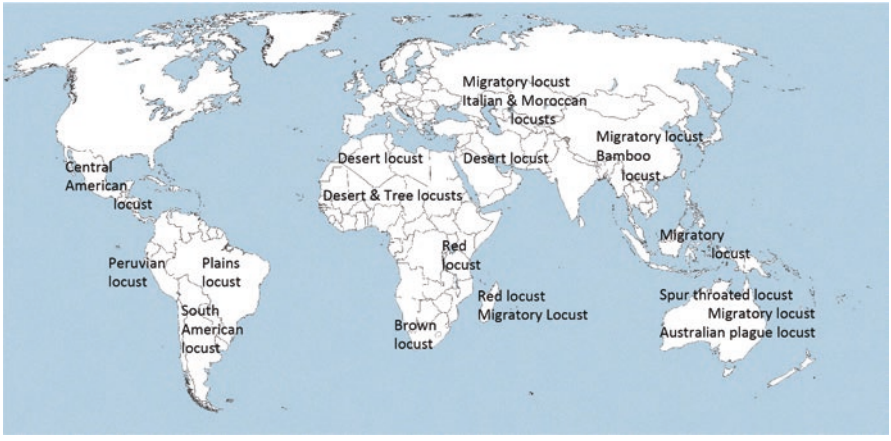

Fig. 2.4 Map of major locusts in world

involve early intervention where government entities that aim to find and treat as many of the locusts as possible *before* they can reach crops.

Economic analysis of the value of early intervention programs demonstrates that such intervention can substantially reduce the economic loss that might otherwise occur. It was estimated the 1984 plague of the Australian plague locust in eastern Australia would have caused $103 million damage (1984 Australian dollars (in 1984 $1A= ~0.90 USD)), if it had not been controlled. Actual damage was $5 million ($98 million dollars less), so that $3.4 million cost of control returned a net benefit of 29:1 on the money invested (Wright 1986). A more recent Australian study showed that concerted control operations conducted by state and national government agencies as well as landholders during the years 2010–2011 had a net benefit of $963 million ($1A = ~1USD). Costs of control operations at all levels were about $50 million giving a benefit-cost ratio of over 18:1 (Millist and Abdalla 2011). Even so, there are times when locusts cause substantial damage to crops: it was reported that, during 2004, the desert locust caused crop losses of >80% in Burkina Faso, Mali and Mauritania with many households requiring food aid (Brader et al. 2006).

The demonstrated value of early intervention programs has meant that there has been an increasing move towards making every effort to find and treat as many of the locusts as possible not only before locusts reach crops but also to begin treatments early in upsurges before locust numbers get too high. It is well recognized that when locusts reach plague proportions, they can cover such large areas and be in such high numbers that they can easily overwhelm national and even international efforts to treat them. Such early intervention programs have had varying degrees of success and some of the most successful have been with species where breeding early in outbreaks is largely limited to localized *outbreak areas* that are particularly favourable for breeding. In studies on locusts with outbreak areas, Uvarov (1921, 1928, 1937) proposed that early intervention while locusts were within outbreak areas would lead to the prevention of plagues. Teams were to monitor and control the low to moderate locust populations in outbreak areas with the view that intensive timely treatments within these areas would prevent high population increases and large scale escapes into cropping areas. And for some locust species with outbreak areas, including the Red Locust *Nomadacris septemfasciata* (Serville) in southern Africa, the African Migratory Locust *Locusta migratoria migratorioides* (Reiche & Fairmaire), and until recently for the South American Locust *Schistocerca cancellata* (Serville), plagues have been prevented for many years (Gastón 1969; Hunter and Cosenzo 1990; Magor et al. 2008).

However, these locusts with outbreak areas are, unfortunately, a special case as most locusts have favoured habitats that are much more widespread, covering hundreds of thousands of km^2. Some can be extremely widespread: the Australian plague locust can be found in an area of more than 2 million km^2, while the desert locust can be found in an area of 16 million km^2, a substantial part of which is not easy to access. Within these large areas, locusts do have particularly favourable habitats (such as open plains for the Australian plague locust (Hunter 1982)) that can be surveyed more intensively, but finding localized bands and swarms in such a

large area can be very difficult, particularly early in outbreaks when populations are small to moderate. Consequently, while early intervention can reduce the size of upsurges and plagues of these widespread species, actual plague prevention is often just not possible. Another complicating factor is that while some locust species are present much of the time in damaging numbers and so require constant monitoring and control, other locusts are in relatively low numbers for long periods leading to a substantial reduction of resources during inter plague periods: when upsurges do occur, the large scale treatments required are delayed until additional resources arrive, by which time the locust populations have increased substantially.

This chapter shall examine various types of locust outbreaks and how some of the latest methods to detect locusts and to control them are being used as part of locust control operations in a number of regions and countries.

3 Management of Locusts with Localized Outbreak Areas

3.1 Red Locust in Tanzania and Neighboring Countries

When favorable conditions allow locust population increase, the locusts commonly come together to form marching bands of nymphs and flying swarms of adults. With some locust species, the regions of initial band and swarm formation are limited to localized *outbreak areas.* As part of a plague prevention program as suggested by Uvarov (1928, 1937), a major effort was made to locate the outbreak areas of the Red Locust (*N. septemfasciata*) in Africa, and a number of such areas were found in Tanzania and neighboring countries. Improvements in control techniques during the 1940s and 1950s provided the tools needed for effective preventive control and concentrating survey and control efforts within outbreak areas was spectacularly successful. There were three Red Locust plagues (1847–1854, 1891–1920 and 1930–1944) before preventive control was introduced and there have been none since. The factors important for locust population increase were studied (Symmons 1964) and regular monitoring within outbreak areas was conducted with bands and swarms treated when they were seen. From time to time environmental conditions have been particularly favourable leading to higher numbers of bands and swarms but extra survey and control efforts have led to successful treatment before plagues could develop.

One of the reasons why preventive control has been so successful with the Red Locust in Tanzania and neighbouring countries is that Red Locusts fit the classification of “sustained eruptions” (Berryman 1987) and “eruptive, spreading outbreaks” (Berryman et al. 1987), which have a stable equilibrium at both low and high densities. Locusts at low densities remain that way until exceptionally favorable weather provides conditions suitable for very successful breeding and populations erupt to reach very high (plague) levels. Before preventive control was implemented, the Red Locust had long lasting plagues, indicating a stable equilibrium at a high den-

sity: once locusts reached high numbers, even normal weather that allowed populations to more or less reproduce themselves, kept populations at or near plague proportions for a number of years. This means that the highly favorable factors leading to upsurges and plagues are not common, and provided extra resources are made available when particularly favorable conditions occur, the increased populations can be suppressed and "escapes" from the outbreak areas by flying swarms minimized. And the swarms that do escape are of small enough size that they in turn can be treated successfully. A recent example of extra resources being made available was during 2009, when the United Nations Food and Agricultural Organization (FAO) used emergency funding that allowed on time treatment bands and swarms in the outbreak areas. While some of the area was treated with chemical pesticides, a biological product Green Muscle® (active ingredient, the naturally occurring fungus *Metarhizium acridum*) replaced the use of chemical pesticides near waterways: important in this locust that is commonly found in flood plains. Through regular monitoring and timely treatments using a variety of products, this species has not reached plague proportions in Tanzania and neighboring countries for many years.

3.2 Locusts in Argentina and Madagascar

Plagues were also prevented for many years for the South American Locust, (*Schistocerca cancellata*) in Argentina. The South American Locust had plagues in 48 of the 58 years from 1897 to 1954, but the introduction of a system of prevention (*la lucha preventiva)* has meant there were no plagues for many years since (Gastón 1969; Hunter and Cosenzo 1990). There is regular survey for locusts in outbreak areas in several provinces in the west of Argentina (Catamarca, La Rioja and environs) with the treatment of any bands and swarms seen. From time to time, conditions are favorable for the production of higher numbers of bands and swarms and extra resources are usually made available. However, a major outbreak, with many swarms, occurred from 2015 to 2017. Swarms were first seen during the winter of 2015 in spite of few locusts being seen in the previous summer and autumn (Senasa 2015), suggesting that early breeding was not detected. Because early breeding was not detected, a much greater effort was required to control the many bands and swarms that resulted.

However, even though the Malagasy Migratory Locust, *Locusta migratoria capito* (Saussure) has outbreak areas, it has been able to reach plague proportions in Madagascar at regular intervals and in the latest plague between 2011 and 2015, several million ha were treated, mostly with chemical pesticides though 366 kg of spores of the fungus *Metarhizium acridum* applied during the 2014–2015 locust season (FAO 2015; see Chaps. 3 and 4). The point is, that while treatments of locusts with outbreak areas can prevent plagues, such success relies on constant monitoring and treatments and rapid access to extra resources during upsurges. If populations do get away and reach plague proportions, then a long treatment program lasting several years may be necessary before populations can be suppressed.

4 Management of Locusts That Are Widespread and Require Treatments in Most Years

Many locusts are not found in localized outbreak areas but their favored habitats, where breeding is more successful, cover substantial areas. Some of these widespread locusts are found in significant numbers somewhere in their favored habitats in most years and to keep numbers in check there are regular programs of survey and control. This type of locust management program has some similarities to many other crop pests in that some treatments are required in most years, increasing to higher levels in years of major outbreaks. As with other crop pests, landholders do treat locusts that directly threaten their crops, but government agencies are usually involved as well since it is widely recognized that locust bands and swarms can cause crop damage very quickly so that treatments by government agencies is also required if substantial crop damage is to be prevented. Government agencies generally aim to treat as many of the locusts as possible *before* they reach crops and when locusts do reach cropping areas, both governments and landholders conduct control measures near or within the crops themselves. Local government officers are commonly involved in actual treatments but there is at least coordination by governments at higher levels with their involvement in actual treatments especially when campaigns are large.

Three examples of these types of these regular control programs will be examined here: the small to moderate programs in the Yucatán of Mexico, the moderate to large programs in China and the large to very large control programs covering millions of hectares in Central Asia.

4.1 Central American Locust in Mexico

The small to moderate treatment programs normally required against the Central American Locust *Schistocerca piceifrons piceifrons* (Walker) in Mexico are really only a step up from the treatment programs against locusts with outbreak areas. The favored areas for breeding of this locust are more extensive than that of locusts with outbreak areas in that its favored habitats are common in a number of states in Mexico ranging from the Yucatán in the south to Tamaulipas in the north. In most years, treatment programs covering a few thousands to tens of thousands of hectares (ha) are required. There is an association of increases in populations of this locust with higher rainfall "El Niño" years (Contreras-Servín 2009), and regular monitoring of locust populations means that such increases are usually detected. Swarms do form in such situations but increased treatment programs are put in place using a variety of chemical pesticides, with some treatments with the biological agent *M. acridum* (Unionyucatan.mx 2010; see Chaps. 3 and 4). Finding and treating all of the infestations quickly is often not possible because swarms can be well hidden in dense forest. These swarms can be very dense (Fig. 2.5) but concerted efforts to

Fig. 2.5 A roosting swarm of the Central American Locust, *Schistocerca piceifrons piceifrons* in the Yucatán, Mexico 2009. (Photo by Jim Conrad)

locate and treat these dense adult swarms during the overwintering adult diapause stage is usually sufficient to reduce the locust population and limit crop damage. The Central American Locust is also found from time to time in other countries in Central America and these require rapid deployment of resources from other pest problems to locusts whenever outbreaks occur and, at times, special programs of training and treatment by international organisations like the United Nations Food and Agricultural Organization (UN FAO).

4.2 Migratory Locust in China

Treatment programs against the Migratory Locust (东亚飞蝗; Dōngyà fēihuáng: East Asia Migratory Locust) have a long history in China: the Emperor of the Tang Dynasty appointed full time officers for locust control in the eighth century (Wang et al. 2003). Plagues of locusts have been a regular feature of Chinese agriculture for centuries, with locusts breeding in the large flood prone areas near rivers and lakes, but a concerted effort of flood mitigation and environmental modification has reduced the size of the areas favorable for breeding. The reduced size of the favorable areas combined with concerted efforts to control locusts when they do appear means that, within China migratory locusts no longer reach plague proportions.

Until recently, it was thought that the major locust species in eastern China was the Oriental Migratory Locust, *Locusta migratoria manilensis* (Meyen), but recent studies indicate that most of eastern China has the Asian Migratory Locust, *Locusta migratoria migratoria* (Linnaeus), with the oriental migratory locust limited to the far south of China (Zhang et al. 2009). Migratory Locusts have outbreaks in parts of eastern China virtually every year. And following the flood mitigation and

environmental modification that reduced the size of the areas favorable for breeding, the remnant favorable areas have been extensively mapped (Zhu 1999). Such remnant favorable areas are regularly monitored each spring/summer by local plant protection officers and, when higher density populations are found, they are treated. Locusts have been treated with a variety of chemical pesticides, but biological agents have become increasingly important as well over 100,000 ha of infestations of locusts and grasshoppers were treated with *M. acridum* or *Paranosema locustae* during 2015 (Wangpeng Shi personal communication). A high level of locust mortality, though over a longer time period than with chemicals, can be obtained both with *Metarhizium* and *Paranosema* (Zhang and Hunter 2005; Ding and Zhang 2009; Fu et al. 2010) and such agents are important in avoiding the use of chemical pesticides against migratory locusts in environmentally sensitive areas, especially near water.

The aim of the regular treatment programs is to limit the formation of the dense swarms that used to be common in the past and in most years this program has been successful in that while some swarms do form, they are soon treated and there have not been swarms in plague proportions for many years. There has been a great deal of recent research on this locust including studies on the very long 1900 year record of locust infestations that have shown that locust numbers are higher when precipitation is less (Tian et al. 2011), which in China, is often associated with El Niño events (Zhang and Li 1999). Until recently, survey and control programs have relied on a large workforce to conduct surveys and treatments but the increasing standard of living in China has meant manpower has become more expensive so using information on the factors that lead to outbreaks has become increasingly important in reducing costs by targeting survey and control to when and where locusts are more likely to be present (Zhang and Hunter 2016).

The Migratory Locust is also found from time to time in other countries in Southern Asia and while these require rapid deployment of resources from other pest problems to locusts whenever outbreaks occur. Both this locust and other locusts such as the Bamboo Locust, *Ceracris kiangsu* Tsai, sometimes require special programs of training and treatment by international organisations like the UN FAO.

4.3 Locusts in Central Asia

In Central Asia, the regular treatment programs are even larger and are against two main locust species: the Moroccan Locust, *Dociostaurus maroccanus* (Thunberg) and the Italian Locust, *Calliptamus italicus italicus* (Linnaeus) that require treatments amounting to millions of hectares each year. Locusts are present somewhere within the larger countries in the Central Asia virtually every year: in the largest country Kazakhstan, more than a million hectares are treated each year while in Uzbekistan treatments of hundreds of thousands of hectares per year are common. Smaller countries in the region may only have locusts from time to time, but regular surveys are conducted to find what locusts are present. The constant monitoring and treatment programs in the larger countries mean that there is a dedicated workforce

involved in locust control. Each spring, local plant protection officers conduct surveys of areas favored by the locusts in their area and, as in China, higher density populations are found and treated. Locusts are treated with a variety of chemical pesticides, often depending on price and whether or not there is local production, though there have been tests with various biological agents such as *M. acridum* and *Beauveria tenella* in recent years (FAO 2014). While these locusts do not normally undertake long distance migrations, they can fly tens of kilometers, which is far enough for locusts in one area to invade other areas, for locusts in pasture areas to invade crops and for locusts in one country to invade another. Trans-boundary movement of locusts has been recognized as a persistent problem and efforts to address it have formed an important part of the FAO program of Locust Watch: Locusts in Caucasus and Central Asia (CCA). Over the past few years, this program has been critical in coordinating efforts in each of the ten countries in the Caucasus and Central Asia and has been introducing standardized techniques for detection of locust infestations and their treatment, which aims for an improved preventive strategy of treating locusts early and so limit damage to crops.

From time to time conditions favor outbreaks of the Asian Migratory Locust (*Locusta migratoria migratoria*) in Central Asia. These outbreaks are most common after declining floods provide large areas of green vegetation along rivers. However, access is difficult after floods so studies have been conducted using satellite imagery to detect the green areas left by receding floods (Sivanpillai and Latchininsky 2007) as a way of determining the size and location of areas potentially favorable for the locust. Plant protection officers, both local and from the central government can conduct surveys concentrating on the favorable green areas. When infestations are found they can be treated as part of limiting the size of any upsurge.

5 Management of Locusts That Are Widespread with Outbreaks from Time to Time

A third major type of locust situation is where locusts are widespread and have major outbreaks or plagues from time to time and the Australian Plague Locust (*Chortoicetes terminifera*) in eastern Australia and the Desert Locust (*Schistocerca gregaria*) in Africa and the Middle East are prime examples of this type of locust. The Australian Plague Locust can be found in an area of more than 2 million km^2 and there are some bands and swarms somewhere in this large area in over half of years, with populations reaching major outbreaks or even plagues once or twice per decade. There are also outbreaks in Western Australia, though less often than in the east. Even more widespread is the Desert Locust, which can be found within an area of about 16 million km^2. There are some bands and swarms of this species somewhere within this large area in about half of years and in the past 30 years, there have been plagues only during 1986–1989 and 2003–2005. These two species require special efforts because of the very large size of the area that needs to be surveyed.

However, populations can vary from very small to very large so these species require a small number of core staff involved in survey and treatment that must be expanded rapidly when locust numbers increase, a major challenge for any control program.

5.1 Australian Plague Locust

The Australian Plague Locust can be found anywhere in much of inland of eastern Australia. Prior to the 1970's, each state (Queensland, New South Wales, South Australia, Victoria) was responsible for its own survey and treatment programs, mainly in their agricultural zones. However, the discovery that the Australian Plague Locust was commonly found in the arid interior (Clark et al. 1969) and that it could migrate hundreds of kilometers overnight from the inland to the agricultural zone (Clark 1969, 1971; Farrow 1977) meant that state-based control operations were almost always going to begin late, *after* locusts invaded a state's agricultural zone. Consequently, to overcome this limitation, a Federal government organization, the Australian Plague Locust Commission (APLC) was set up.

The APLC manages locusts through an Integrated Pest Management program using chemical control agents as well as the biological product Green Guard® with the active ingredient a local strain of the fungus *M. acridum*. The control program has a strategy of early intervention, in that treatments begin with the first bands and swarms that form in the interior or agricultural zone, and continue every generation thereafter. The aim is not to treat the whole population but to reduce its size so that the numbers of locusts reaching crops is much less.

To locate and treat the populations that appear early in outbreaks, there is only a small staff of seven to ten field officers to survey the more than 2 million km^2 of inland eastern Australia and they rely on an understanding of where locusts are more likely to be present. Australian Plague Locusts are more often found in open plains (Clark 1969), which can be identified using satellite imagery (McCulloch and Hunter 1983). And locusts are further localised in being most common in open plains that have received rain: areas of more likely rain can be identified using Bureau of Meteorology rainfall interpolations. As well, locust maturation and laying usually occurs in areas where there is green vegetation produced by recent rain, but because there is limited rainfall (200–500 mm/year) in the areas where locusts are found, they usually have to migrate to reach rain areas (Hunter et al. 1981). There is a slight summer maximum in the subtropical inland and a slight winter maximum in the more temperate agricultural zone and locusts tend to migrate in a circuit: there tends to be northwards migrations in late spring/early summer to the summer rainfall areas in the subtropics with autumn return migrations southwards to reach the temperate winter rainfall areas (Deveson et al. 2005). Migrations of hundreds of kilometers are common and at times migrations can cover a total distance of 1000 km or more.

In about half of years, rain is not common and the locusts only have two generations per year, with the two generations of breeding balanced by some mortality

during dry periods, leading to only minor outbreaks with at most localised bands and swarms. However, from time to time rainfall is much more regular leading to three or even four generations in rapid succession over a period of a year or so, then populations increase rapidly. Early intervention relies on being able to rapidly locate and control the significant locust infestations that result when rain is regular and finding localized locust infestations within the large area of eastern Australia has been aided by the use of a Decision Support System (DSS) for locust management that helps estimate where and when locust infestations are more likely to be (Deveson and Hunter 2002). The DSS is computer based and consists of layers of information, starting with a base map of the open plains habitats favoured by locusts (Clark 1969; Hunter 1982; McCulloch and Hunter 1983). Onto the base map can be placed most recent locust distributions, as well as distributions during past outbreaks back to 1970. Then the distribution of recent rain and directions of likely locust migrations are mapped using rainfall interpolations and upper level wind data from the Bureau of Meteorology. After locusts have reached the adult stage, accumulation of fat reserves required for migration (Hunter et al. 1981) can be estimated by computer models and by sampling of locusts for fat reserves and forecasts of timing of migrations made. Following forecast migration events, surveys are concentrated in the areas more likely to be invaded as part of locating any swarms that are present. When swarm invasion and subsequent maturation and oviposition are detected or suspected, development models for embryonic and nymphal development are run so surveys can be conducted when and where nymphal bands are likely to be present. Surveys are conducted by ground vehicle but once upsurges are underway, bands can be so dense and so large that they can be seen from an aircraft flying overhead (Fig. 2.6). Survey using aircraft has proven a valuable part of rapidly detecting locust infestations and delineating areas of bands suitable for spraying (Hunter et al. 2008).

And once locusts are detected, they are treated with a variety of techniques. Chemical pesticides are important both in blanket treatments often with fenitrothon, and with treatment in strips using the slightly more persistent fipronil (Hunter 2004). However, the increasing constraints on chemical insecticide use have led to the use of a biological agent, the fungus *M. acridum.* This biological alterative has been used operationally since the 2000–2001 locust season, when nearly 25,000 ha of locust bands were treated, with nearly 100,000 ha used in the years since. Its use on organic properties, in national parks, near waterways and where there are rare and endangered species ensures that locusts can be treated wherever they are found, important in ensuring the success of the early intervention strategy of managing locusts.

Of course, even with this variety of techniques, plagues can sometimes occur. Early localized populations can easily be missed and even when they are found, treatment can be hampered by widespread rainfall and/or flooding, which is relatively common during the regular rains that lead to upsurges and plagues (Hunter 2004). If early treatments are missed, locusts can then be found in so many areas that only some can be treated. Even so, early intervention is valuable in reducing the size of the upsurge/plague and so reducing the damage that can occur when locusts

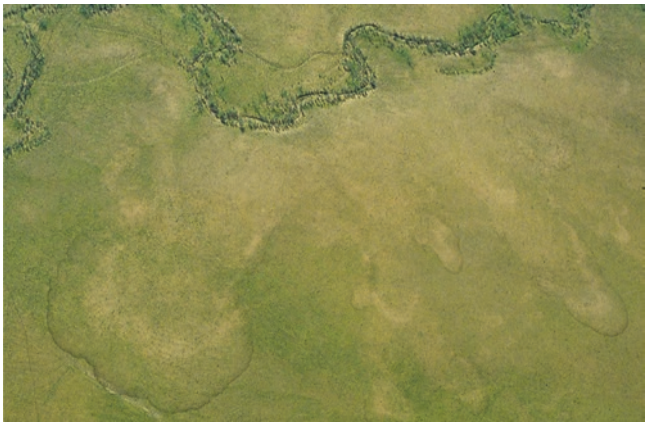

Fig. 2.6 Bands of the Australian Plague Locust *Chortoicetes terminifera* seen from an aircraft flying overhead at a height of 400 m. Note the curved black band front of a >500 m long band and a number of smaller bands and the pale areas of eaten vegetation

reach crops. And when large numbers of locusts are in the agricultural zone, there is substantial control by not only by the APLC but also by local authorities and landholders somewhat like occurs in the larger countries of Central Asia most of the time; the extra effort helps keep crop damage to a minimum. And the lessons learned in the Australian experience with the use of satellite imagery, the various components of a DSS and integration of both chemical and biological pesticides have been introduced in other countries with similar problems and such exchange of techniques will become increasingly important as various regions face the yet uncertain effects of climate change on locust outbreaks.

5.2 Desert Locust

The Desert Locust, *S. gregaria* can be found within an even larger geographical area (~16 million km^2) that extends from Mauritania/Morocco in west Africa eastwards through Arabia to the Rajasthan Desert in India (Uvarov 1957; Waloff 1966), making it a world renowned severe pest (see Fig. 2.4). The Desert Locust has a migratory circuit with long distance migrations covering hundreds or even thousands of kilometers between areas that tend to get rain in summer and those where rain falls more often in winter. In about half of years there is enough rain somewhere in the vast recession area for localised bands or swarms to form but rain is not common in much of the area so that any localised increases for a generation or so are usually balanced by dry periods where migrating locusts do not reach a rain area, leading to population decline. However, from time to time there are very favourable conditions consisting of a sequence of rains in both the summer and winter rainfall areas over a year or so leading to a rapid population upsurge and even to plagues. Breeding

sequences leading to plagues are not common: there have been only two plagues in the past 30 years and these have only lasted a few years (1986–1989 and 2003–2005).

By contrast, plagues of the Desert Locust in the first half of the twentieth century were long lasting (8–14 years). The long lasting nature of these plagues reflects the eruptive nature (Berryman 1987) of desert locust outbreaks, where a period of very favourable conditions led to an upsurge to plague levels and once plague levels were reached, even normal conditions were able to maintain populations at or near plague levels. In recent years, Desert Locusts have sometimes still been able to reach plague proportions during very favourable periods. But such favorable periods usually last only a year or so and when weather conditions return to normal and population increase is much less, a substantial international control effort that involves treatments covering millions of hectares, by the FAO and other international organizations has been is able to suppress the locust population so that recent plagues have been short lived, lasting only a year or two. Even so, even these shorter plagues can be devastating: during 2004, the desert locust caused crop losses of >80% in Burkina Faso, Mali and Mauritania with many households requiring food aid (Brader et al. 2006).

The control strategy for the desert locust is one of early intervention/preventive control where areas where locusts are likely to breed successfully are surveyed and infestations detected. In recent years, delimiting areas where rain has fallen and locusts might breed has improved through the use of satellite imagery to estimate recent rainfall and assess vegetation response (Cressman 2013). Within areas where rain has fallen, surveys can be concentrated in a structured way in areas of favored habitat (Sword et al. 2010) and some countries have effective survey programs in that favoured habitats are regularly surveyed and locust infestations detected. However, actually preventing plagues of the Desert Locust has proven very difficult because locating and treating a substantial part of upsurge populations is often not possible. One of the major limitations of detecting and controlling Desert Locusts is the reliance on ground survey to locate and treat bands. In much of the Desert Locust area, good roads are few, and off road travel difficult, such that it is often not possible to locate and treat the thousands of bands present, particularly during summer when nymphal development takes just over a month. The result is that even in countries with good survey and control programs, there is not enough time to find and treat most of the bands and swarms early in upsurges. And effective preventive treatments just do not occur in countries whose survey and treatment programs are limited by a lack of resources or to factors such as insurgencies that make access virtually impossible. And a further compounding factor is that from time to time, *substantial* extra resources are required at short notice to combat the rapidly increasing upsurge populations that occur when conditions are particularly favourable. The substantial damage that can occur has prompted many national international government and humanitarian organizations to support desert locust control but the requirement for large amounts of money and resources at short notice means the extra resources required often arrive late *after* the upsurge is well underway. One reason for the delay is that organizations tend to have most of their funds committed early in a financial year making it difficult to find the extra resources required if

locusts suddenly erupt. The second problem is the massive amounts of resources required: it was estimated that international donors contributed USD 274 million to the 1986–1989 Desert Locust control campaign (Skaf et al. 1990).

However, preventive control programmes are the control strategy utilized by the FAO to combat the desert locust and the limitations to preventive control were outlined by Magor et al. (2008) and emphasized even more strongly for the 2003–2005 outbreak by Symmons (2009). But it must be recognized that the concerted international efforts that occurred once the upsurge was well underway was able to suppress the plague which means that plagues are now much shorter in duration than they once were and their shorter duration and much reduced extent has largely delivered the original objective of Uvarov (1951) in preventing damage to the major agricultural zones in the invasion area.

6 Remarks and Conclusions

The most successful preventive control programs are those that have a combination of at least some understanding of how upsurges and plagues originate and sufficient resources for a rapid response as upsurges are occurring so that locust infestations can be located and then rapidly treated using a variety of techniques. This combination of efforts is necessary for early intervention, and is able to reduce the size of locust populations before they can damage crops and can even prevent some plagues (Magor et al. 2008). Early intervention *can* lead to plague prevention especially for species with certain characteristics that make them more amenable for control, namely they have localized outbreak areas that can be monitored regularly and where there is ready access to the extra resources required when upsurges do occur. The Red locust in Tanzania and neighbouring countries is a good example of this, as has the South American locust in Argentina, at least until recently. Locust populations can also be limited with species that have outbreaks most of the time: then officers can be dedicated to survey and control with additional officers and resources quickly made available when populations increase. Preventive control programs have been quite successful in limiting damage in places like China where there are locusts requiring treatment on a regular basis and so there are substantial resources available that can be expanded when infestations become larger. However, for some other locust species, prevention of plagues has proven difficult. Species that only have outbreaks from time to time usually have only a small workforce involved in their monitoring and control and when upsurges occur, there is often not enough extra resources made available quickly to keep populations in check. And as Symmons (2009) indicated, with species like the Desert Locust that are widespread and found in remote areas, such early intervention is rarely if ever actually prevents plagues; a substantial effort can rapidly reduce locust numbers once plagues start and adequate resources are made available on an international scale.

However, we need a close examination of what is trying to be achieved with programs of early intervention. The commonly used term in English, preventive

control, has a connotation of plague prevention in the minds of many. However, in both French (*la lutte préventive*) and Spanish (*la lucha preventiva),* the literal meaning of the words is really closer to a preventive "struggle" or "battle" against the locusts that does not necessarily have a connotation of prevention of plagues. The aim of *la lutte/la lucha* early intervention treatments is to reduce the size of invasions of crops and, while *la lutte préventive* might be able to prevent some plagues, particularly in certain species, reducing crop damage is the real economic benefit of this strategy. Early intervention, rightly applied with adequate resources, is able to lead to a substantial reduction in crop damage and economic loss.

7 The Future of Locust Control

The increasing constraints both on the use of chemical insecticides and on methods of control mean that continuing success, let alone any further improvements, will require continuing research development and operational testing in all aspects of locust control. Constraints arise not only from the need to continually reduce effects on the environment, but also because of a desire by many countries to have little or no chemical residues in the food products they import. There are further constraints resulting from increasing standards of operator health and safety that mean methods used formerly are now severely restricted or curtailed. Some of this research will involve examining products and methods that have been used successfully in other control programs and FAO has been instrumental in introducing techniques used elsewhere to control infestations in Central Asia and in Africa. Further work needs to be done on improved methods of locating infestations, which for some locusts will mean the use of satellite imagery to detect where rain has fallen and habitats are suitable for locust increase and, for species with dense bands, detecting locust bands using aircraft (Hunter et al. 2008; Latchininsky 2013). There needs to be continuing studies on developing and using alternatives to currently used chemical pesticides, including biopesticides, insect growth regulators, and even attractants and pheromones, though the latter have had limited success for locusts despite substantial research efforts. Only then can continued and even more importantly improved success be guaranteed in a world of increasing human population and climate change where protection of crops from damage will be a vitally important part of maintaining food security.

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Chapter 3
Effects of Pesticides on the Environment and Insecticide Resistance

Gaelle Le Goff and Maeva Giraudo

Abstract The fight against pest insects has become a major challenge nowadays to eliminate disease vectors such as malaria, dengue fever or Zika virus, and to grow healthy crops to be able to feed a constantly increasing world population. Insecticides represent one of the main solutions to this challenge but with the introduction of every new insecticide comes inevitably the apparition of resistance a few years later. This chapter provides an overview of the evolution of the different insecticide families over time and their effects on the environment. Resistance mechanisms involving target modification and increased metabolism are detailed for each chemical family. The recent emergence of other resistance mechanisms such as the modification of insect cuticle permeability, the role of ABC transporters in xenobiotic excretion, and the involvement of symbionts are also discussed.

1 Insecticide Evolution and Mode of Action

1.1 A Brief History of Insecticide Discovery

Before the 1850s, the use of inorganic insecticides such as arsenic and boric acid prevailed, offering only a marginal control of pest insects. The development and expertise in chemical synthesis later allowed the massive use of insecticide to control pest insects in crops. Then came the discovery of plant derivatives such as nicotine, rotenone and pyrethrum, and in the 1920s, the knowledge and understanding of the structure and synthesis of these compounds, which opened the way to organic synthetic pesticides (Fig. 3.1).

Chemists were able to modify the structure of the compound to increase its persistence and potency, and the discovery of new synthetic organic insecticides

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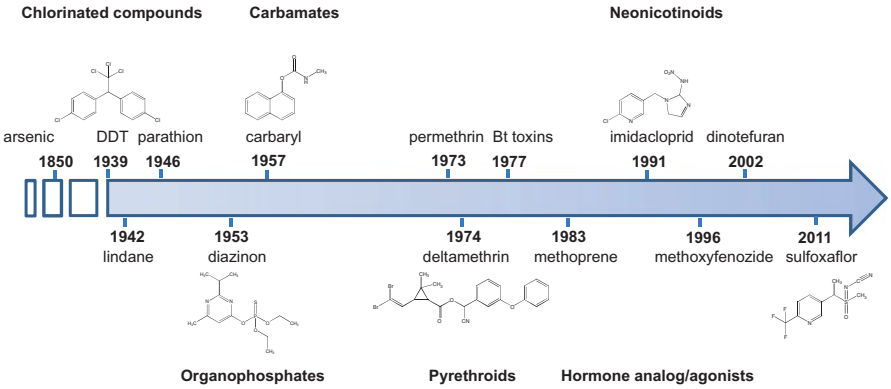

Fig. 3.1 Dates for the discovery of the main classes of insecticides with some important molecules

became the main goal of the 1930s. In 1939, Müller identified the insecticidal activity of **DDT**, although Zeidler had originally discovered this compound in 1874. This led to the development of **chlorinated compounds**, and to the discovery in 1942 of the insecticidal action of lindane and hexachlorocyclohexane (Casida and Quistad 1998), quickly followed by aldrin, dieldrin, and endosulfan. These chlorinated insecticides played a major role in insect control between 1940 and 1970 but after 1970, their use was drastically reduced or banned in some parts of the world (e.g., in the USA, France, and the UK), while less persistent compounds were released on the market. In the meantime, **organophosphates** (OPs) were developed, with the discovery of the first OP in 1937 by Schrader. Since then, the phosphorylation of common molecular motifs led to the development of many OPs with insecticidal activity such as parathion, malathion, and diazinon (Fig. 3.1). Synthetic **carbamates** development began in 1947, with the two most famous and well-developed compounds carbaryl and aldicarb. These substances are synthetic derivatives of major legume alkaloids such as physostigmine or eserine.

Pyrethroids appeared in the early 1970s, including permethrin in 1973 and deltamethrin in 1974, and were more potent at lower doses than those required for OPs or carbamates. However, the risks associated with organic insecticides for human and environmental health shifted the interest towards the use of more natural compounds. Biopesticides appeared in the 1970s with the discovery of the insecticidal activity of the ***Bacillus thuringiensis*** (Bt) endotoxin (Goldberg and Margalit 1977), even though the first report of Bt was made in 1902 in diseased *Bombyx mori* (Melo et al. 2016). Special interests were also focused on the development of insecticides that can mimic the action of growth and developmental hormones, i.e. 20-hydroxyecdysone (20E) and juvenile hormone (JH). This approach led to the discovery of many insecticide compounds, including fenoxycarb, **bisacylhydrazines**, and methoprene, which was discovered in 1983 (Hsu 1991), and more recently methoxyfenozide (Le et al. 1996). In the 1990s–2000s, **neonicotinoids** were developed in an attempt to use compounds with high specificity for insect

pests, low vertebrate toxicity and environmental persistence, and high biodegradability potential. Neonicotonoids are derived from natural compounds such as nicotine and epibatidine. Imidacloprid was the first neonicotinoid commercialized in 1991, followed by many others such as thiacloprid in 2000 and dinotefuran in 2002 (Bass et al. 2015). Neonicotinoids are currently the most widely used insecticides in the world and represented 26% of the total insecticide market in 2010 (Sparks 2013). The other classes of insecticides developed at the same time such as spinosyns, diamides, avermectins, and fiproles never reached these levels of production and use (Sparks 2013). Developing new chemicals with different modes of action represent a real challenge and the agrochemical industry is constantly working on the discovery of new insecticides.

A safer alternative to insecticides for successful pest control is the perturbation of insect olfactory system (see this book volume 2). Insects are highly dependent on olfaction cues to find their host plants as well as to locate and mate with their sexual partner. Sex pheromones are indeed the major players in insect mating communication. The first isolation and synthesis of an insect pheromone was reported at the end of the 1950s by Butenandt and collaborators in *Bombyx mori* (Butenandt et al. 1959). From then, it was suggested that this type of molecules could be exploited as pest control agent with the advantage of being highly specific towards the targeted insect species (Wright 1964). The knowledge and improvement of insect pheromones synthesis then paved the way for their use in pest control. Pheromone-based pest control is promised a bright future because of the reduced number of authorized insecticides, the establishment of new regulations, and the increasing interest for more sustainable molecules. After the big wave in insecticide discovery between the 1940s and the 1970s, the marketing of new insecticides has significantly decreased for two main reasons. First, insecticide development costs have increased due to new regulations, which now require between 8 and 12 years of preliminary assessment before a new molecule can be distributed. Today, these costs represent 256 million dollars per molecule (Galm and Sparks 2016). In addition, the number of products that are evaluated before obtaining a marketable compound was multiplied by ×100 in 60 years (Sparks 2013). Second, insecticides must meet several requirements before commercialization, including enhanced selectivity and activity, low risks for environmental and human health, and high biodegradability potential.

1.2 Insecticide Mode of Action

The majority of insecticides act by disrupting the activity of the insect nervous system (Fig. 3.2). Although all insecticides might not have the same target, the symptoms are often similar and can be described in four consecutive phases: excitement, convulsion, paralysis and death.

The toxic effect of **DDT** and **pyrethroids** is mediated through preventing the closure of voltage-dependent sodium channels that are involved in the transmission of action potential (Lombet et al. 1988). This mechanism results in the continuous

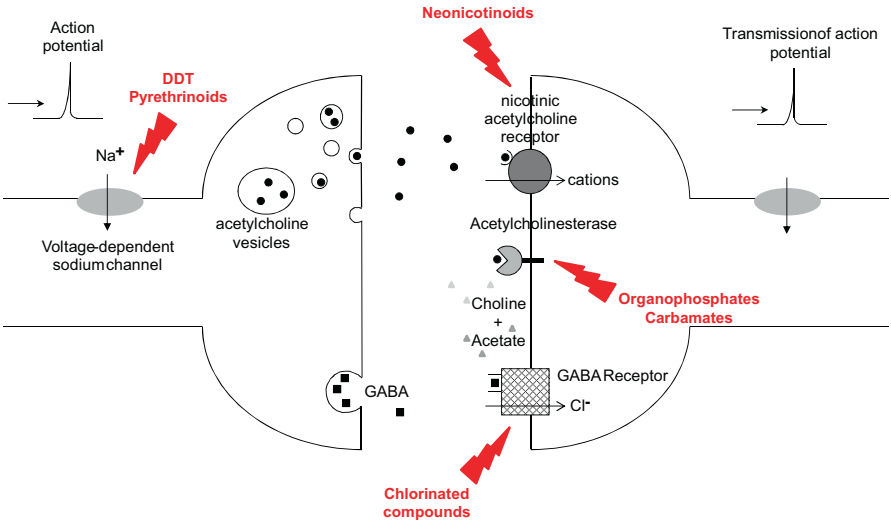

Fig. 3.2 Main targets of insecticides at a synapse

activation of sodium channels and delays the normal inactivation mechanism due to voltage (Soderlund and Bloomquist 1989). The visible effect in insects is called the "knock-down", which corresponds to an initial and often transitional paralysis.

Non-DDT **chlorinated compounds** such as cyclodienes act on the chloride channels γ-aminobutyric acid (GABA) receptors involved in the desensitization. The binding of neurotransmitters to the GABA receptors provokes the opening of the channel and the increase of the chloride conductance, which ultimately leads to the inhibition of a new action potential. Insecticides are also able to bind to the GABA receptor and inhibit the Cl^- flux (Lawrence and Casida 1983; Bloomquist and Soderlund 1985). Similarly to pyrethroids, these compounds act on the insect nervous system and hyper excitation symptoms precede insect death.

Organophosphates and **carbamates** inhibit acetylcholinesterase (AchE), which controls nervous influx transmission by breaking down acetylcholine into the inactive products choline and acetic acid. Insecticides bind to the active site of the enzyme at the same site of acetylcholine, which is no longer hydrolyzed, causing a hyper stimulation of the cholinergic system and the same symptoms as described above (Fukuto 1990).

Bt toxins are encapsulated inside a crystal that can be ingested by insects. These parasporal crystal inclusions are solubilized in the gut and proteins are released as δ-endotoxins. These protoxins are then activated by intestinal proteases, bind to membrane receptors located in the gut epithelium, and cause pores inside the membrane, which lead to the death of the insect (Palma et al. 2014).

Bisacylhydrazines are agonists of the growth hormone 20E and induce lethal molts with symptoms similar to an excess of ecdysteroids (Williams 1967). Although 20E regulates the expression of many genes, some genes are only expressed after a drop in 20E levels prior to molting, hence the developmental disruption by biacylhydrazines resulting in insect death.

Neonicotinoids act on the nicotinic acetylcholine receptors (nAChR) present in the post-synaptic neuron. Under normal condition, acetylcholine binds to those receptors, which causes nervous stimulation. The return to the steady state is provided by AchE, which breaks down acetylcholine. The binding of neonicotinoids to nAChR is irreversible and provokes a high level of activation of these receptors, which leads to insect paralysis followed by death (Nishimura et al. 1998; Nishiwaki et al. 2003).

2 Effects of Insecticides on the Environment

The introduction and use of synthetic insecticides began in the 1920s, but it was not until the 1960s that the first environmental impacts were reported by Rachel Carson in her famous book « Silent Spring » in which she documented the detrimental effects of pesticides on the environment, especially on birds. One of the most notable example was the dramatic impact of the organochlorine DDT and its metabolite DDE on the decrease in eggshell weight in birds of preys (Ratcliffe 1967). These observations led to a national ban of DDT in the US and since then, huge efforts have been made to develop less persistent and more specific substances to control insect pests. Despite an increased specificity, the large scale use of potent and persistent insecticides has raised serious concerns about their risk for the environment and non-target species affected by these chemical compounds. The potential for leaching, spray drift and runoff into surface waters is one of the major concerns surrounding extensive use of insecticides on agricultural fields, especially those in close proximity to water bodies.

Since the first reports of environmental impacts of DDT in the 1960s, **organochlorines** (OCs) have been extensively studied due to their high persistence, biomagnification potential through the food chain, and their highly bioaccumulative nature (Chopra et al. 2011). Compounds such as endosulfan and DDE have been detected in many environmental compartments and biota despite their ban in most countries. The presence of lindane and endosulfan was reported as far as in arctic waters, where bioconcentration factors of endosulfan were higher in zooplankton and various fish species than in more temperate environments (Weber et al. 2010). OCs act mostly as endocrine disruptors in fish as reviewed in Senthilkumaran (2015), and a comparative study of the effects of pesticides in honeybees reported that endosulfan significantly decreased bees olfactory learning performances (Decourtye et al. 2005).

Organophosphate insecticides act by inhibiting the AchE activity in insect nervous system and therefore, AchE activity has been used widely in terrestrial and aquatic non-target species as a biomarker of exposure and effect of OPs. Although OPs are relatively non persistent and rapidly degraded in the environment, they have broad-spectrum specificity and show high acute toxicity towards non-target vertebrate and invertebrate species. Up to 90% inhibition of AchE activity were reported in fish inhabiting streams polluted by OPs (Fulton and Key 2001). The OP

chlorpyrifos has been particularly studied in recent years after being associated with massive fish kills in the US in the late 1990s. Studies in fish showed that chlorpyrifos affected mostly behavioral responses normally associated with the AchE activity in the nervous system such as swimming behavior (Giddings et al. 2014). In addition, a recent study showed that low levels of chlorpyrifos measured in wild honeybees from New Zealand were able to severely affect formation and retrieval of appetitive olfactory memories (Urlacher et al. 2016).

Carbamate insecticides are frequently detected in freshwaters and carbaryl has recently been reported at concentrations of up to 950 ng/l in surface waters of southern Ontario in Canada (Struger et al. 2016). These concentrations are higher than the lowest observed effect concentrations (LOEC) affecting the cumulative number of molts and neonates in the freshwater crustacean *Daphnia magna* (Toumi et al. 2016). Carbamates such as carbosulfan are also able to inhibit AchE activity in fish similarly to OPs and these effects are increased synergistically by a co-exposure to the OPs malathion and triazophos (Wang et al. 2015a).

Synthetic **pyrethroids** are used in pest control all over the world and have low toxicity to mammals and birds, however they are known for exerting toxic effects towards honeybees, freshwater fish and different aquatic organisms. The toxic response of fish to pyrethroids such as deltamethrin has been extensively studied using a variety of biomarkers as reviewed in Kaviraj and Gupta (2014). Results showed that these compounds affect different molecular and cellular pathways such as oxidative stress, energy metabolism, and induce genotoxicity. Moreover, pyrethroids have been incriminated along with neonicotinoids as the main chemical factors causing the global decline of honeybee populations over the world. Compounds such as cypermethrin and permethrin caused locomotor deficiency and impairment of detection and processing information at different levels of the bee olfactory system (Kadala et al. 2014; Charreton et al. 2015).

The production of genetically engineered crops producing the insecticidal crystal Cry proteins from **Bt** have increased over the last few decades. The development and use of Bt as insecticide were motivated by the high selectivity of the Cry toxin towards specific insect orders. A large numbers of studies have indicated low levels of hazard to most groups of non-target organisms, as reviewed in Clark et al. (2005). Dietary exposure to very high doses was necessary to induce negative effects on body size and fecundity of *D. magna* exposed to BT-maize leaves (Holderbaum et al. 2015). No effects were observed on survival, pollen consumption and olfaction abilities of honeybees exposed to Cry1 toxins, nor did it cause toxicity in honeybee larvae (Wang et al. 2015b; Dai et al. 2016). The effects reported in non-target species seem to be the result of synergism with extrinsic factors such as other insecticides rather than a direct effect of Bt toxins (Then 2010).

Analogues of the molting hormone 20E and JH are among the most potent insecticides. These compounds target specifically endocrine pathways of insects and most arthropods. Data on non-target effects in vertebrates such as fish or birds are almost inexistent in the literature. However, effects of hormone analogues have been reported in non-target arthropods such as the freshwater crustacean *D. magna*, where the JH analogues fenoxycarb and epofenonane impacted signaling pathways involved in molting and development (Toyota et al. 2014).

Neonicotinoids are persistent, have low volatility, and are quite soluble in water (up to 4.1 g/l for thiametoxam). Even though these compounds were banned in most European countries, they are still in use in North America where concentrations at the µg/l level have been reported in streams and surface waters. These concentrations are in the same range than the LC_{50} values measured in aquatic insects and crustaceans as reviewed in Anderson et al. (2015). In a recent study, Gibbons et al. (2015) reviewed more than 150 published studies on the toxic effects of neonicotinoids on non-target vertebrate species such as fish, birds, mammals, amphibians and reptiles. They found that both imidacloprid and clothianidin exerted toxic effects by impairing reproduction, growth and immune functions in all species studied at sub-lethal concentrations. Most importantly, neonicotinoids have been clearly identified as one of the main causing factors of the massive losses of honeybees colonies that happened in 2006 (Fairbrother et al. 2014). More recently, it was shown that low doses of imidacloprid and thiamethoxam impaired short-term olfactory memory in the same species (Wright et al. 2015).

3 Insecticide Resistance, the Two Main Mechanisms: Modification of the Target and Metabolic Resistance

Insecticide resistance has been defined by the Insecticide Resistance Action Committee as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species". Resistance is not a new fact, the first case of resistant insects has been reported in 1914 in response to an inorganic insecticide (Melander 1914). The effort put into insect pest control through the introduction of new insecticide families has always been overcome by the emergence of resistance 2–20 years later (Fig. 3.3).

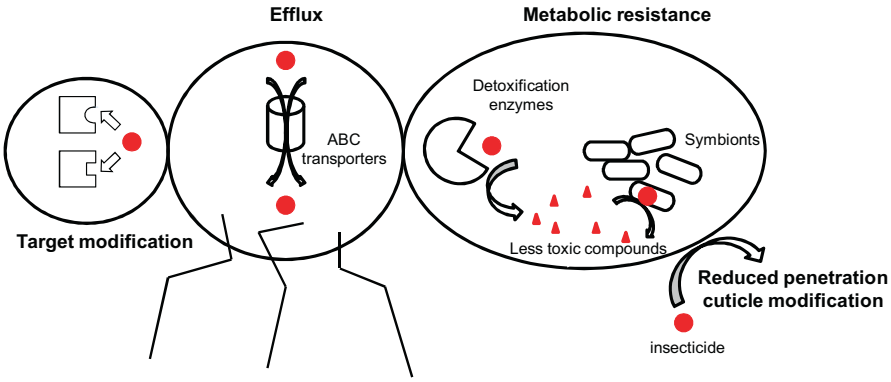

Fig. 3.3 Main resistance mechanisms

3.1 Modification of the Target

Most insecticides target proteins essential to nervous system functions, which leads to major constraints for insects. Indeed, insect become resistant through protein modifications without affecting the endogenous function of the protein. The following paragraphs will focus on the main insecticide targets cited previously (Fig. 3.4).

3.1.1 Voltage-Dependent Sodium Channel

The voltage-dependent sodium channel "*para*" (for paralytic temperature sensitive) was initially cloned in *Drosophila melanogaster* (Loughney et al. 1989). This channel is made of four homologous domains (I–IV) structurally organized to form a pore in the center, with each domain is consisting of six transmembrane segments (S1–S6) (Fig. 3.4a). The first mutation that conferred resistance to DDT and pyrethroids was identified as the replacement of leucine 1014 to phenylalanine and was located in the sixth transmembrane segment of the second domain (IIS6). This mutation was named kdr for "knock-down resistance" and was initially found in *Musca domestica* (Williamson et al. 1996). Many other studies have revealed the presence of the same mutation in other resistant species such as *Blatella germanica* (Miyazaki et al. 1996; Dong 1997), *Anopheles gambiae* (Martinez-Torres et al. 1998), and *Myzus persicae* (Martinez-Torres et al. 1999). The L1014F substitution

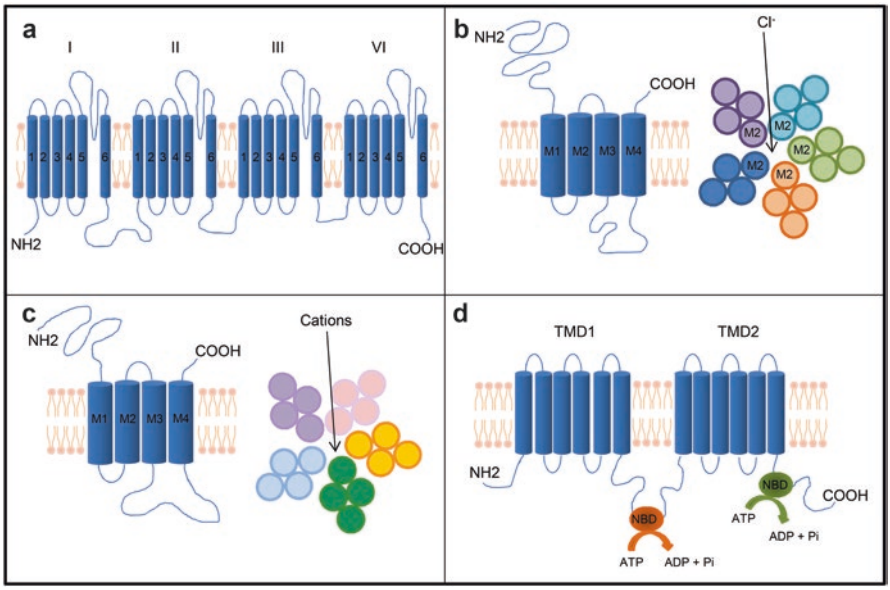

Fig. 3.4 Structure of (**a**) voltage-dependent sodium channel, (**b**) GABA receptor, (**c**) Nicotinic acetylcholine receptor, (**d**) ABC transporter

results in the reduction of the open state, which is the preferred state for pyrethroid insecticides (Vais et al. 2003; Davies et al. 2008). Furthermore, the mutation would induce a conformational change of the sixth transmembrane hydrophobic segment, which could affect the insecticide (Tan et al. 2005). The same position has also been found with other substituted amino acids leading to L1014C/H/S/W (Rinkevich et al. 2013). An additional mutation named super-kdr was found in *M. domestica,* which replaced the methionine 918 by a threonine in the cytoplasmic loop between the fourth and fifth transmembrane segment of the domain II (Williamson et al. 1996). The association of kdr and super-kdr confers high level of resistance to pyrethroids.

After the initial discovery of these two mutations, other mutations at different positions have been reported to be involved in the resistance to DDT and pyrethroids. For example, in *D. melanogaster* four other mutations conferred insecticide resistance through the reduced affinity of the channel for pyrethroids (Pittendrigh et al. 1997; Martin et al. 2000). In the cockroach, the F1538I mutation found in the IIIS6 completely abolished the sensitivity to several pyrethroids (Tan et al. 2005). In a recent review, Rinkevich and collaborators have reported the existence of 30 different mutations present in at least two species and around 20 mutations present in only one species (Rinkevich et al. 2013), which underlines the diversity of sequence modifications that can confer insecticide resistance.

3.1.2 GABA Receptor

The GABA receptor ensures fast inhibitory synaptic transmission by converting the binding of GABA in a rapid and transient increase in permeability to chloride ions. This receptor is a membrane protein with four transmembrane segments and a large extracellular N-terminal domain. The channel is formed by the assembly of five monomers of this type, the second transmembrane domain of each of these proteins forming the wall of the channel (Fig. 3.4b).

The gene encoding the GABA receptor was initially cloned in a resistant strain of *D. melanogaster* and named *Rdl* for "resistance to dieldrin" (Ffrench-Constant and Roush 1991). A point mutation corresponding to the substitution of A301S in the second transmembrane segment was found in resistant strains (Ffrench-Constant et al. 1993). The same substitution was found in many other insect species [see (Feyereisen et al. 2015) for a review]. The replacement of alanine 301 by a glycine was also found in *D. simulans*, *An. gambiae*, several species of *Anopheles,* and in *M. persicae* (Thompson et al. 1993; Anthony et al. 1998; Du et al. 2005; Le Goff et al. 2005; Asih et al. 2012), or by an asparagin in rice planthoppers such as *Laodelphax striatellus* and *Sogatella furcifera* (Nakao et al. 2010, 2011; Nascimento et al. 2015). The A301S mutation has two effects: first, it alters the binding site of the insecticide, and second, it helps to destabilize the desensitized state of the GABA receptor (Zhang et al. 1994). The GABA receptor has a different binding site than cyclodienes, which has helped selecting a mutation that affects only the binding of insecticides without disrupting the GABA binding and the general function of the channel.

In addition to conferring resistance to cyclodiene, A301S/G/N confers also a cross-resistance to the more recent compound fipronil. In combination with this substitution, additional T350M or R357Q mutations have been reported in fipronil resistance (Le Goff et al. 2005; Nakao et al. 2010, 2011). In the brown planthopper *Nilaparvata lugens*, A301S and Q359E are associated to ethiprole resistance (Garrood et al. 2017). A duplication of the Rdl locus was found in two resistant species (Anthony et al. 1998; Remnant et al. 2013). In the aphid *M. persicae* resistant to endosulfan, one locus had the A301S and the other locus carried either the wild type A301 or the A301G (Anthony et al. 1998). In *D. melanogaster* resistant to dieldrin, one locus had the wild type A301 and the second locus beared the A301S mutation (Remnant et al. 2013). Authors have suggested that the benefit to have two copies of *Rdl* would allow maintaining the endogenous function while conferring resistance. Moreover, gene duplication contributes to the increase in the amount of GABA receptor expressed, which is also an important element in insecticide resistance.

3.1.3 Acetylcholinesterase

The contribution of AChE in insecticide resistance is species-specific. Indeed, the majority of insects has two copies of AChE (*Ace-1* and *-2* genes coding for AChE1 and AChE2, respectively), while higher dipteran such as *D. melanogaster* and *M. domestica* only have *Ace-2*. It was suggested that these dipteran had lost the *Ace-1* gene during evolution (Weill et al. 2002; Huchard et al. 2006).

The gene *Ace-1* was found for the first time in the genome of *An. gambiae* (Weill et al. 2002), while the gene *Ace-2* was initially cloned in *D. melanogaster* (Hall and Spierer 1986). Several mutations have been identified in higher dipterans that contributed to resistance against OPs and carbamates. At least nine different positions in *Ace-2* gene have been reported to confer resistance, and some of them with could be substituted by several amino acids like G227A or V (Fournier et al. 1992; Mutero et al. 1994; Kozaki et al. 2001; Walsh et al. 2001; Vontas et al. 2002; Menozzi et al. 2004). Some of these mutations reduced insecticide access to the enzyme catalytic site (Mutero et al. 1994; Walsh et al. 2001; Russell et al. 2004). In addition to these identified positions in higher dipterans, three other positions in *Ace-2* have been involved in insecticide resistance in other insects (Zhu et al. 1996; Nabeshima et al. 2004; Chen et al. 2007). However, AChE1 is considered to be the main catalytic enzyme for most insects and potentially the main target for insecticides (Revuelta et al. 2009; Kim and Lee 2013).

A G119S substitution was identified in populations of *Culex pipiens* from different geographic origins (Africa, Caribbean and Europe) and conferred resistance to propoxur (Weill et al. 2003). The same mutation was reported in two other mosquito species, *An. gambiae* and *An. albimanus* (Weill et al. 2003, 2004). Modeling analysis revealed that the mutation was located in the active site of the enzyme (Weill et al. 2003). At least six additional positions were involved in resistance and certain positions were conserved between species like the S331F in aphids such as

M. persicae and *Aphis gossypii* and F331W in the sweetpotato whitefly *Bemisia tabaci*, which was also located in the active site gorge (Benting and Nauen 2004; Nabeshima et al. 2004; Alon et al. 2008).

Duplication of *Ace-1* has been reported in several mosquito species (Bourguet et al. 1996; Labbé et al. 2007; Djogbenou et al. 2008, 2009). In the main vector of Malaria, the mosquito *An. gambiae*, populations from West Africa carried one copy of the susceptible *Ace-1* and another with the mutation G119S, which conferred resistance. The association of both susceptible and resistant alleles could lead to a rapid spread of the resistance because it induce a selective advantage by reducing most or all of the fitness cost due to the presence of the mutation (Assogba et al. 2015). The duplication is largely spread and was found in 173 field collected resistant mosquitoes from several African countries (Assogba et al. 2016). The mosquitoes had either susceptible and resistant alleles or two resistant alleles. Duplication of *Ace-2* has also been suggested in *A. gossypii* resistant to OP (Shang et al. 2014).

3.1.4 Nicotinic Acetylcholine Receptor

The nicotinic acetylcholine receptor (nAChR) acts on the synaptic cholinergic transmission. This receptor is formed by the assembly of five subunits with a cation-permeable channel in the center. Each subunit corresponds to a protein with an N-terminal extracellular domain, four transmembrane domains and a large loop between the third and fourth transmembrane domains (Fig. 3.4c). The genome of *D. melanogaster* revealed the presence of ten genes coding for nAChR subunits, seven α-subunits and three β-subunits [for a review see (Sattelle et al. 2005)]. The discrimination between α and β is based on the presence of an YXCC motif in the α subunit, which is involved in the binding of acetylcholine. Different subunits can be assembled to form different subtypes of nAChRs with their own characteristics of conductance, transmitter affinity, ionic selectivity or pharmacology. AchRs are the target of spinosyns and neonicotinoids. It has been demonstrated that the binding site for spinosyns was different from the one for neonicotinoids (Salgado and Saar 2004; Orr et al. 2009; Puinean et al. 2013), which resulted in a different modification to confer resistance. For spinosyns, most of the identified mutations were present in the α-6 subunit. The first case of spinosad resistance was reported in *D. melanogaster* and was associated to a loss of function due to a mutation-induced truncated form of the α-6 subunit (Perry et al. 2007).

Later on, several mutations have been reported to cause a mis-splicing and create a premature stop codon in the sequence, leading to a truncated protein. This was the case for the diamond back moth *Plutella xylostella* and the oriental fruit fly *Bactrocera dorsalis* (Baxter et al. 2010; Rinkevich et al. 2010; Hsu et al. 2012). More recently, the presence of a punctual G275E mutation in the α-6 subunit was found to be associated with spinosad resistance in different insect species such as the melon thrips, *Thrips palmi* (Bao et al. 2014), the western flower thrip *Frankliniella occidentalis* (Puinean et al. 2013), and the tomato leaf miner moth *Tuta absoluta* (Silva et al. 2016). The introduction of this mutation by CRISPR/Cas9 in *Drosophila*

demonstrated its implication in resistance to spinosad (Zimmer et al. 2016). Homology modeling analyses suggested that the mutation was located at the top of the third α-helical transmembrane domain of the α-6 subunit (Puinean et al. 2010), and that it might be involved in the binding of the insecticide.

Moreover, a new spinosad resistance mechanism involving exon skipping was recently identified in *T. absoluta* (Berger et al. 2016). In the resistant strain, the SpinSel exon3 of nAChR α-6 was missing, and this exon was demonstrated to be essential for the sensitivity to spinosad. It was suggested that this exon skipping mechanism might be due to epigenetic modifications (Berger et al. 2016). It is worth noting that the large variety of α-6 modifications enables insecticide resistance because this subunit is not essential for insect survival.

In the case of neonicotinoids, distinct mutations have been shown to respond to resistance. For instance, Y151S point mutation was found in the two nAChR α-1 and α-3 subunits in *N. lugens* (Liu et al. 2005). The binding of imidacloprid was significantly reduced in *Xenopus* oocytes when nAChR expressed these subunits (Liu et al. 2005). Another R81T substitution in the α-1 subunit was also reported in *M. persicae* (Bass et al. 2011; Slater et al. 2011) and *A. gossypii* (Koo et al. 2014). This mutation was located in the loop D of β-1 and was potentially involved in the binding of neonicotinoid insecticides and acetylcholine (Grutter and Changeux 2001; Shimomura et al. 2006).

3.2 Metabolic Resistance

Most insecticides are hydrophobic molecules that are metabolized in the organism into more hydrophilic compounds, which can be readily excreted. Changes in expression levels and catalytic activities of enzymes involved in insecticide metabolism enable the development of resistance. When studying the mechanism of metabolic resistance, the first step usually involves the use of specific inhibitors of each major detoxification enzyme family such as cytochrome P450s, glutathione S-transferases, and carboxylesterases. For instance, a higher toxicity observed in the presence of the P450 inhibitor piperonil butoxide would suggest a P450-induced resistance.

In addition, the use of model substrates can result in higher enzyme activities, which can help identifying resistant species. Further studies are then required to identify the specific gene responsible for the resistance amidst those multigenic families. The final step would involve functional studies to demonstrate the ability of the enzyme to metabolize the compound into a less toxic compound. There are many examples for each detoxification enzyme and many good reviews on the subject, therefore the following paragraphs will illustrate each enzyme family by focusing on one particular resistant gene.

3.2.1 Cytochrome P450

The first case of P450-dependent resistance was reported in 1960 in the housefly; the use of the P450 inhibitor sesamex was able to suppress the resistance to carbamates (Eldefrawi et al. 1960). Many examples of P450-induced resistance have since then been documented for different insecticide families and for a variety of insect species such as *Drosophila* (Daborn et al. 2002), the moth crop pest *Helicoverpa armigera* (Joussen et al. 2012), the aphid *M. persicae* (Puinean et al. 2010), and the insect vectors Mosquitoes (David et al. 2013). Most studies showed a correlation between P450s overexpression and insecticide resistance, thanks to the advent of molecular biology techniques such as microarrays (DNA chips) in the early 2000s, and the more recent high-throughput sequencing. These analyses result in a list of candidate genes, which need to be complemented by functional studies to demonstrate the specific involvement of P450s in the resistance.

One example is *Cyp6g1* from *D. melanogaster*, whose overexpression in transgenic flies was sufficient to obtain a strain resistant to DDT and imidacloprid (Daborn et al. 2002; Le Goff and Hilliou 2017). This overexpression was the result of the insertion of an *Accord* transposable element in the promoter region of *Cyp6g1*, and was found in many populations around the world (Daborn et al. 2002; Catania et al. 2004). A duplication of the genomic region was also identified in resistant flies and contributed to a higher expression level of the transcript (Schmidt et al. 2010). Heterologous expression of CYP6G1 in tobacco cell culture demonstrated the capacity of the enzyme to effectively metabolize imidacloprid and DDT (Joussen et al. 2008). Additional modeling analysis suggested that despite a relatively small catalytic site, at least six insecticides (i.e., imidacloprid, DDT, nitempyram, acetamiprid, malathion and N-phenylthiourea) could be docked inside the CYP6G1 3D structure (Jones et al. 2010).

3.2.2 Glutathione S-Transferase

The first case of insecticide metabolization by a GST was reported in 1966 for OP compounds (Fukami and Shishido 1966). Similarly to P450s, GSTs can confer resistance to different insecticide families. For example, the epsilon 2 GST (GSTe2) from mosquitoes has been shown to be involved in the resistance to DDT and pyrethroids in many studies. In *An. gambiae*, GSTe2 is over-expressed in laboratory selected strain (David et al. 2005) as well as in field collected strains (Djegbe et al. 2014; Mitchell et al. 2014). Heterologous expression of GSTe2 in *Escherichia coli* was able to metabolize DDT (Ortelli et al. 2003).

The analysis of the promoter region of *GSTe2* revealed a two adenosine indel, which was responsible for the increased transcription level in the resistant strain (Ding et al. 2005). In *An. funestus*, a highly resistant strain to DDT and permethrin,

GSTe2 was also the most overexpressed detoxification gene. In addition to overexpression, Riveron and collaborators have also demonstrated that the introduction of *GSTe2* with the point mutation L119F in transgenic *Drosophila* was sufficient to confer resistance to permethrin (Riveron et al. 2014). Three dimensional analysis revealed that the mutation was able to increase the access to the active site, which resulted in an increased enzyme activity and a high level of resistance (Riveron et al. 2014). In addition, another I114T mutation of *GSTe2* identified in *An. gambiae* was able to increase the level of DDT resistance in transgenic *Drosophila* (Mitchell et al. 2014).

In the yellow fever mosquito *Aedes aegypti* from Africa and Thailand (see Chap. 1), GSTe2 overexpression was reported in DDT and pyrethroids resistant strains (Lumjuan et al. 2005, 2011). Further analyses demonstrated the ability of GSTe2 to metabolize DDT, and the RNA interference-induced knockdown of *GSTe2* expression caused an increased susceptibility to deltamethrin (Lumjuan et al. 2005, 2011).

3.2.3 Carboxylesterase

The involvement of carboxylesterases in insecticide resistance has been demonstrated by using synergist compounds such as S,S,S-tributyl phosphorothioate (DEF), triphenyl phosphate (TPP) and S-benzyl O,O-diisopropylphosphorothionate (IBP) (Apperson and Georghiou 1975; Georghiou et al. 1980; Hemingway and Karunaratne 1998).

Two main mechanisms are generally distinguished; one corresponds to insecticide sequestration, and the second to increased catalytic activity. In the first case, gene amplification (Mouches et al. 1986; Field et al. 1988) and/or gene regulation (Rooker et al. 1996; Kwon et al. 2014) increase the expression of the protein, which will rapidly bind the insecticide but slowly release the metabolites, resulting in sequestration of the molecule (Karunaratne et al. 1993). In the second mechanism, point mutations in the coding genes reduce the carboxylesterase activity but allow the parallel acquisition of OP hydrolase activities. These activity changes are responsible for OP resistance in some strains of *M. domestica* and *Lucilia cuprina,* formely named *Phaenicia cuprina,* the green bottle or Australian sheep blowfly (Campbell et al. 1997; Newcomb et al. 1997; Claudianos et al. 1999).

Esterases can confer resistance to different families of insecticides such as OPs, carbamates and pyrethroids. One of the most well-known mechanisms of gene amplification is the case of carboxylesterases E4/FE4 in *M. persicae* (Devonshire and Field 1991). In resistant strains of *M. persicae*, these two carboxylesterases were involved in the elimination of the insecticide by both hydrolysis and sequestration through the overproduction of carboxylesterase resulting from gene amplification. It was suggested that a succession of tandem duplications of the carboxylesterase E4 gene was associated with the resistance (Devonshire and Sawicki 1979).

The gradual increase in the amount of esterase E4 was correlated with the level of resistance observed in a series of aphid clones. The direct evidence of the involvement of E4 and FE4 gene amplification in resistance was obtained using Southern

blot comparison of DNA from susceptible and resistant aphids (Field et al. 1993). The carboxylesterase genes were amplified by up to 80 times and overproduced esterases represented up to 3% of the total proteins in the most resistant aphids (Field et al. 1996, 1999).

4 More Recent Mechanisms

The emergence of new technologies such as Next Generation Sequencing (NGS) has led to a renewed interest for specific mechanisms and gene families that have been given relatively less attention in insecticide resistance. The following paragraphs explore the recent and growing interest in the role of cuticular proteins, ABC transporters and symbionts in resistance (see Figs. 3.3 and 3.4).

4.1 Insect Cuticle Modification

Insect cuticle represents one of the main entry points for insecticides along with the respiratory system and the digestive tract. A modification of the cuticle decreases the insecticide penetration rate, leading to an increased degradation of insecticide by metabolic detoxification. This mechanism only confers high level of resistance when it is associated with other synergistic mechanisms such as an increased detoxification (also see Chap. 9, volume 2).

The first cases of resistance involving a reduced penetration of the insecticide were reported 50–60 years ago but were not given much attention for several years. The emergence of NGS has helped bringing to light this mechanism as a possible cause of insecticide resistance. An increase in the expression of some cuticular proteins has been found in several cases of resistance. The first studies reporting a reduced insecticide absorption in resistant insect strains were mostly based on the measurement of the amount of radiolabeled insecticide present at a given time inside and outside the insect. This mechanism has been highlighted in the house fly *M. domestica* (Fine et al. 1963; Sawicki and Farnham 1968), the common house or northern house mosquito *C. pipiens* (Stone and Brown 1969), the red flour beetle *Tribolium castaneum* (Walter and Price 1989) and the tobacco budworm *Heliothis virescens* (Lee et al. 1989). However, only a few studies were conducted at that time to understand the molecular mechanisms involved.

The advent of microarrays and RNA sequencing has later revealed that the level of transcripts coding for cuticular proteins were elevated in several resistant insect species, especially in mosquitoes in response to pyrethroids. In *An. coluzzii* (formerly known as *A. gambiae* M molecular form) and the malaria vector *An. arabiensis*, the same transcripts coding for a Cuticular Protein Analogous to Perithrophin (CPAP3-A1b) were elevated in the resistant strains (Nkya et al. 2014; Toe et al. 2015). These proteins are known to maintain the structural integrity of the cuticle.

Using scanning electron microscopy, Wood et al. (2010) for example showed that the cuticule was thicker in permethrin resistant strains of *An. Funestus,* one of the major malaria vectors in Africa (See Chap. 1). The enzyme laccase 2 involved in the sclerotization and pigmentation of the insect cuticle was found overexpressed in *C. pipiens pallens* resistant to fenvalerate (Pan et al. 2009). The authors suggested that this enzyme could participate in the hardening of the cuticle, which led to a reduced rate of insecticide penetration. While reduced penetration is associated to pyrethroid resistance in mosquitoes, it confers resistance to additional insecticide families in other species, such as DDT in *D. melanogaster* (Strycharz et al. 2013) or neonicotinoids in the aphid *M. persicae* (Puinean et al. 2010). Only a few studies however have gone further in the understanding of this reduced insecticide penetration or its regulation.

Drosophila is probably the most well-studied insect model. For example, it was shown that the high level of resistance against DDT of the strain 91-R was multifactorial and partly due to a reduced insecticide penetration. Observations of *Drosophila* adult cuticle using electron microscopy showed differences between resistant and susceptible strains, with a more laminated structure and a greater thickness for the strain 91-R. The composition of cuticular hydrocarbons was common to both strains, but a higher quantity of several hydrocarbons was present in 91-R (Strycharz et al. 2013). Several candidate genes have been tested by RNA interference to explain those differences in cuticle structure and reduced penetration of DDT, such as the larvae cuticular protein *Lcp1,* which is over-expressed in 91-R (Strycharz et al. 2013), and the cytochrome P450 *Cyp4g1*, which is involved in the last step of the formation of cuticular hydrocarbons (Qiu et al. 2012). In the UAS-RNAi lines, the knockdown of these genes induced a significant increase of the DDT susceptibility (Gellatly et al. 2015). Therefore *Lcp1* and *Cyp4g1* play a role in DDT resistance probably by reducing insecticide penetration.

The role of other CYP4G in reduced penetration of insecticide has also been suggested in resistant population of *Anopheles*. Overexpression of *Cyp4g16* and *Cyp4g17* was found in pyrethroids resistant mosquitoes associated with an increased cuticle thickness and higher cuticular hydrocarbon content (Balabanidou et al. 2016). The regulation of many cuticle genes has also been reported to be dependent of the transcription factor CncC, which is known in *Drosophila* to be the main regulator of the xenobiotic response (Misra et al. 2011).

4.2 ABC Transporters

ATP-binding cassette transporters (ABC transporters), are involved in xenobiotic detoxification mechanisms. They are present in all living organisms from bacteria to human and are involved in the transport of a wide range of compounds such as amino acids, sugars, lipids, and peptides.

A functional transporter is composed of two transmembrane domains (TMDs) with six transmembrane segments and two cytosolic nucleotide-binding domains

(NBDs) that can bind and hydrolyze ATP (Fig. 3.4d). These proteins use the energy from ATP hydrolysis to transport substrates across cell membranes. ABC transporters are classified into eight different families from the letter A to H based on similarities in their ATP binding domain. In eukaryotes, they function as pumps to excrete toxins and drugs out of the cell. Although ABC transporters have been extensively studied in human, especially for their role in multidrug resistance in cancer therapy (Dlugosz and Janecka 2016), they were relatively ignored in insect until recently.

The number of studies published on the role of ABC transporters in insecticide resistance has significantly increased in the last few years as reviewed in Dernmauw and Van Leeuwen (2014). ABC transporters are involved in the resistance against all families of chemical insecticides, including organophosphates, carbamates, pyrethroids and neonicotinoids. An increased level of transcripts coding for ABC transporters has been observed in many resistant insect species. In mosquitoes, *ABCB4* is over-expressed in *Ae. aegypti* resistant to pyrethroids (Bariami et al. 2012), *ABCG4* and *ABCB1* in *An. arabiens* resistant to DDT (Jones et al. 2012) and *ABCA1*, *ABCB4* and *ABCA4* in *An. gambiae* resistant to pyrethroids and DDT (Fossog Tene et al. 2013).

The expression of some ABC transporters could be induced by insecticides, suggesting their potential implication in insecticide resistance. In *Anopheles stephensi*, the major vector of human malaria in Middle East (see Chap. 1), *ABCG4* was induced after exposure to permethrin (Epis et al. 2014). However, the mechanisms and pathways involved in the insecticide resistance mediated by ABC transporters are largely unknown. Synergistic effects of the ABC inhibitor verapamil (a calcium channel blocker) have been shown for several mosquito species, including the dengue vector *Ae. aegypti* in response to temephos (Lima et al. 2014), *An. stephensi* exposed to permethrin (Epis et al. 2014), and *C. pipiens* in response to cypermethrin, endosulfan and ivermectin (Buss et al. 2002). The importance of a specific ABC transporter in insecticide resistance was investigated using RNAi experiment in *Ae. aegypti* and showed that the silencing of *ABCB1* gene increased the toxicity of temephos (Figueira-Mansur et al. 2013).

In addition, ABC transporters can also confer resistance to Bt toxins. The first demonstration was done in the cotton pest *H. virescens* (Gahan et al. 2010). In this species as well as in *P. xylostella* and *Trichoplusia ni*, the resistance to Cry1Ac was associated to the gene *ABCC2* and for *H. virescens* and *P. xylostella* to a truncated form of the protein (Gahan et al. 2010; Baxter et al. 2011). A down-regulation of *ABCC2* expression was found in resistant strains of *P. xylostella* (Lei et al. 2014; Guo et al. 2015). In addition, the suppression of *ABCC2* expression by RNAi in susceptible strains decreased significantly the susceptibility to the toxin. Authors showed that *ABCC2* and *ABCC3* down-regulation was controlled by the Mitogen-Activated Protein Kinase (MAPK) signaling pathway (Guo et al. 2015). However, the key transcription factor working downstream the MAPK signaling cascade and directly involved in the regulation of the ABCC2 transporter still remains to be identified.

In *B. mori,* a very interesting study showed that a single amino acid insertion in ABCC2 was able to confer resistance to Cry1Ab. Authors used transgenesis to show

that the removal of the insertion of a tyrosine (Y234) in the outer loop of the predicted transmembrane structure, transformed the resistant strain into a susceptible one (Atsumi et al. 2012). The heterologous expression of ABCC2 with the Y234 modification in Sf9 cells demonstrated a lack of Cry1Ab binding to the transporter and a reduced susceptibility of the cells to Cry1Ab and Cry1Ac compared to the cells expressing ABCC2 without the Y234 (Tanaka et al. 2013).

4.3 Symbionts

There is a growing interest in the scientific community to understand the interactions between species and particularly the influence of microbiotes on higher organisms. In insects, the protecting role of the symbiotic bacteria *Hamiltonella defensa* was demonstrated in the aphid *Acyrthosiphon pisum* against its natural enemy, the parasitoid wasp *Aphidius ervi* (Oliver et al. 2003). The beneficial effect of this endosymbiont is due to its ability to produce a toxin-encoding bacteriophage (Oliver et al. 2009). In the case of insecticide resistance, the influence of the symbiotic bacterium is both positive and negative. The presence of *Rickettsia* in the sweetpotato whitefly *B. tabaci* induced an increase in the susceptibility to some insecticides including acetamiprid, thiamethoxan and spiromesifen (Kontsedalov et al. 2008). On the contrary, the beneficial effect of the symbiont has been recently demonstrated in the bean bug *Riptortus pedestris* (Kikuchi et al. 2012). When this insect lived in symbiotic association with the bacteria from the genus *Burkholderia,* its life cycle was shorter and its body size increased compared to uninfected insects (Kikuchi et al. 2007). Acquisition of the bacteria occurs in each generation via the environment (Kikuchi et al. 2007). The bacteria *Burkholderia* is found in free-living organisms in the soil and it is possible to find Burkholderia resistant to insecticide in agricultural fields where insecticide treatments have been applied. Some strains have been demonstrated to be able to degrade the organophosphate fenitrothion and use it as a carbon source (Hayatsu et al. 2000; Tago et al. 2006). Kikuchi and collaborators have looked at whether the bacteria capable of metabolizing fenitrothion could be found in symbiotic associations with *R. pedestris* and confer resistance (Kikuchi et al. 2012). In laboratory experiments, they have shown that repeated treatments of the soil by insecticide increased the proportion of resistant bacteria and that insects harboring resistant bacteria became resistant to fenitrothion. However, they were not able to isolate symbiotic bacteria with the ability to metabolize insecticide in field samples of *R. pedestris,* nor in the rice bug *Leptocorisa chinensis*, which is also known to be found in association with *Burkholderia* (Kikuchi et al. 2005, 2011). Nevertheless, in sugarcane fields where fenitrothion was massively used such as in the Japan Island Minami Daito, they found that the oriental chinch bug *Cavelerius saccharivorus* was living in symbiosis with fenitrothion-degrading *Burkholderia* (Kikuchi et al. 2012).

This kind of resistance mechanism could be quickly acquired and spread and should be taking into account in insect pest management programs in the future.

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Chapter 4
Discovery and Development of Microbial Biological Control Agents

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Abstract The use of microbial biocontrol agents as biopesticides has become an attractive insect pest management choice due to concerns regarding effects of chemical pesticide residues on human and animal health. In the biopesticide approach, an endemic microbial pathogen of an insect pest is identified that is capable of rapidly infecting and killing the target pest when the focal area is inundated. Initial screenings focus on finding aggressive pathogens of the target insect pest. The host range of these pathogens is then evaluated to ensure their safety. Formulations are subsequently developed to ensure effective application in the field. Once developed, the microbial biocontrol agent is used in a manner similar to a chemical pesticide. The mode of action, however, is such that the infective propagules produce multiple infections in the insect pest resulting in death. Because the biocontrol agent is restricted in its host range, only microbial propagules which contact and infect susceptible pests survive. Long-term persistence in the environment normally does not occur, so typically the inundation approach to microbial biocontrol does not produce multi-year control of the pest. Thus, seasonal application of the biocontrol agent is required, which provides the commercial incentive for development.

1 Why Develop Microbial Biocontrol Agents?

The use of microbial biological control (or simply 'biocontrol') agents as biopesticides is an especially attractive pest management choice to counter concerns regarding effects of chemical pesticide residues on human and animal health as well as the environment. In the biopesticide approach, an endemic microbial pathogen of an insect pest is identified that is capable of rapidly infecting and killing the target pest

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when the focal area is inundated. Initial screenings of potential biopesticides focus on finding aggressive pathogens of the target insect pest. The host range of these pathogens is then evaluated to ensure their safety. Formulations are subsequently developed to ensure effective application of the biopesticide in the field. Once developed, these microbial biopesticides are used in a manner similar to chemical pesticides. The mode of action, however, is such that the infective biopesticidal propagules produce multiple infections in the insect resulting in death. Since the biopesticide agent is restricted in its host range, only microbial propagules which contact and infect susceptible pests survive. Long-term persistence in the environment does not occur normally, so typically the inundation approach to biocontrol rarely produces multi-year control of the pest. Thus, seasonal application of the biopesticide is required, which provides the commercial incentive for development of these products.

2 Agent Development, Production and Deployment

2.1 Fungal Biocontrol Propagules

Insect pest management with microbial pathogens has gained popularity mostly due to the success of *Bacillus thuringiensis* (Bt)-based products. While bacteria are often appropriate candidates for use as biopesticide agents for control of certain foraging insects, fungal pathogens frequently show the best potential for development as generalized insect control agents. Hundreds of fungal pathogens of insects have been identified as candidate biopesticides. The ability of fungal pathogens to actively infect and kill insect pests is a unique quality that promotes their widespread use as commercial biopesticides. While fungal pathogens do not require that the host defenses be compromised for infection to occur, they do require contact with the host pest and free moisture to ensure germ tube growth and penetration into the host. Once inside the target insect, the fungal pathogen spreads throughout the tissue consuming nutrients. When the insect host dies, the fungus consumes the remaining nutrients and may erupt from the host tissue to sporulate on its surface, thus providing infective propagules for other individuals that are nearby. Under optimal conditions epidemics or epizootics may occur in target insect populations. In addition, the hyphae of the fungal pathogen may differentiate within the host to form desiccation-tolerant sclerotial bodies. These compact, hyphal aggregates serve as survival structures for the fungus and can be large (sclerotia) or small (microsclerotia). Following environmental cues, sclerotia are capable of hyphal, sporogenic (conidial spore production) or carpogenic (fruiting body formation) germination in the following growing season, thus providing inoculum for infection of the pest target.

The availability of a cost-effective method for mass-producing and formulating stable, infective propagules is an overriding factor when selecting microbial biocontrol agents for commercial development as biopesticides (Stowell et al. 1989, 1991; Jackson et al. 1997, 2010; Wraight et al. 2001; Jackson 2007). Following the development of the pharmaceutical industry in the 1940s, the use of liquid culture fermentation has been the method of choice for the industrial production of microbial products. The production of fungal biopesticides for insect and weed control follows this trend with over 60% of the currently available agents being produced using deep-tank fermentation (Copping 2004). In fact, of the ten insect and weed fungal biocontrol agents produced and sold in the United States, eight are produced using liquid culture fermentation (Copping 2004). In addition to economic factors such as methods of commercial production, selecting fungal biocontrol agents for commercial development must take biological factors into consideration. An understanding of the life-cycles of the target pest and the fungal pathogen being developed as a biocontrol agent is essential if suitable fungal propagules are to be targeted for production and use as a biopesticide (Vega et al. 2009; Jackson et al. 2010; Adiyaman et al. 2011; Schisler et al. 2014). This ecological information can then be used to determine if a suitable fungal form can be produced using liquid culture techniques (Jackson and Schisler 2002; Vega et al. 2003; Shearer and Jackson 2006; Jackson and Jaronski 2009), as it represents the most cost-effective method for producing large quantities of microbial propagules (Stowell 1991; Grimm 2001).

Fungal biocontrol agents that are used as granular applications in soil or aquatic environments need to be very stable propagules. Conidia and "yeast-like" propagules are often poor candidates for these applications (Daigle et al. 1998; Jaronski and Jackson 2008). Conversely, fungal propagules such as microsclerotia or chlamydospores that function as survival structures for many fungi are well suited for use as granular biopesticides. We have shown that when the plant pathogen *Colletotrichum truncatum* is grown in liquid media containing a high concentration of carbon, dense vegetative growth is followed by the formation of high concentrations of microsclerotia (Jackson and Schisler 1994). Microsclerotia of *C. truncatum* are extremely stable as dry preparations, germinated in hyphal and sporogenic fashion, infected and killed emerging hemp sesbania seedlings, and remained viable when used as seed coatings or soil amendments (Schisler and Jackson 1996; Boyette et al. 2007). It was demonstrated that the fungal entomopathogen *Metarhizium anisopliae* produced high concentrations of microsclerotia under specific nutritional conditions during liquid culture fermentation (Jaronski and Jackson 2008). These microsclerotia were desiccation tolerant with excellent storage stability following air-drying. When air-dried microsclerotial granules of *M. anisopliae* were plated on water agar or soil, they produced infective conidia via sporogenic germination following rehydration that infected and killed the sugar beet root maggot (Jaronski and Jackson 2008). The potential of microsclerotial preparations of *M. anisopliae* to deliver an infective conidial inoculum in situ represents a significant improvement compared to the use of spore- or mycelium-based granules applied to soil.

2.2 Fungal Propagule Stabilization and Formulation

Fungal propagules that are produced using liquid culture fermentation are hydrated and metabolically active. Drying is a preferred method for the long-term stabilization of these living microbial agents. On a commercial scale, three basic methods of drying can be used: spray drying, air drying and freeze drying. High temperatures and the rapid removal of water are physical conditions which are often detrimental to cell survival during drying (Fu and Etzel 1995), although recent breakthroughs in spray drying blastospores of *Beauveria bassiana* and *M. anisopliae* suggest that this approach to drying may be plausible (Kassa et al. 2004; Jin and Custis 2013; Mascarin et al. 2016). The term "air drying" is used in this context to describe cell drying techniques which incorporate cooler drying temperatures and longer drying times. In this process, cells in the fermentation culture broth are generally mixed with a filter-aid, de-watered, placed in shallow pans and slowly dried with ambient temperature air flow (Mary et al. 1985; Jackson and Schisler 1994). The air drying process can require 6–20 h and be performed in various commercial dryers such as tumbler dryers, rotating blade dryers, tray dryers and fluidized bed dryers. Air-drying studies with blastospores of *Isaria fumosorosea* showed that the relative humidity of the drying air influences the long-term storage stability of dried blastospore preparations (Jackson and Payne 2007). In general, a final moisture content of 1–4% has been shown to enhance spore survival during storage. Air dryers are relatively inexpensive to operate and provide an economical method for drying and stabilizing living, microbial preparations.

Formulation technology is integral to the success of any biopesticide project. The goals of formulation include making the product safer and easier to handle, improving storage stability, improving persistence of the microbial agent when applied, and enhancing the infectivity of the agent by providing exogenous nutrients or by improving contact with the target (Wraight et al. 2001; Knowles 2008). Current formulation technology is highly segmented among microbial biocontrol agents owing to the specific requirements of each agent and the differing target pests and environments. Not only are microbial biocontrol agents represented by diverse classes (bacteria, viruses, and fungi), but within a single fungal organism, there exists multiple propagule forms (conidia, blastospores, microsclerotia, hyphae) with differing properties. When considering the number of diverse combinations of organisms, propagules, target pests, and control environments, there are endless permutations in the development of specific formulations to address these unique parameters. This diversity dictates that the development of a formulation strategy must be integrated with the development of the production and stabilization technologies to ensure that the final product possesses the characteristics necessary to deliver consistent pest control under expected field conditions.

One of the ways a formulation can improve the efficacy of a fungal biocontrol agent is to improve the delivery and attachment of the active propagule to the target

pest. From a technical perspective, these include technology or formulation practices that improve the dispersion, spatial delivery and adhesion of the microbial propagule to its target. Most of the biopesticide products currently available are formulated to facilitate their application as a sprayed liquid suspension of propagules. Examples of these products include wettable powders, water-dispersible granules, liquid/suspension concentrates, and emulsions. Formulation strategies to improve the delivery and attachment for liquid microbial applications have been broadly applied. For example, surfactants and wetting agents can be used to improve the dispersion of microbial propagules in spray suspension (Jin et al. 2008). The spatial delivery of biopesticides can be improved by formulating the propagule as a foam-forming suspension (Dunlap et al. 2007). Adhesion of the liquid droplets and microbial propagules can be altered by manipulating the dynamic surface tension and interfacial surface tension with adjuvants (Forster et al. 2005).

The discovery of liquid culture fermentation methods for producing microsclerotia of insect, weed, and plant disease biocontrol fungal agents provides an opportunity for the development of new biopesticide products for use in difficult pest control environments, such as soil and water applications (Jackson and Schisler 1994; Shearer and Jackson 2006; Jackson and Jaronski 2009; Kobori et al. 2015). Sclerotia are unique fungal propagules because they are capable of producing an infective conidial inoculum in situ. Faria et al. (2009) published a comprehensive list of mycoinsecticides and mycoacaracides. Most products are based on solid substrate-produced conidia as the active propagule and target pests are predominantly arthropods that feed on the leaf and stem portions of plants, with a limited number of products/microbes targeting pests in soil (scarab larvae, termites) or aquatic environments (mosquito larvae). The development of sclerotium-based formulations will broaden the use and improve the efficacy of these fungal biopesticide agents in soil and water, where spray application of conidial preparations is ineffective (Behle et al. 2013).

3 Challenges to Production and Field Deployment

The major challenges that face widespread adoption of biopesticide use in a “real world” setting mostly center around the application and efficacy of the fungal agent in the field. Although the ability of fungal entomopathogens to successfully kill a broad array of insect pests has been demonstrated a plethora of times in the published literature, most of these studies have been performed either in laboratories or in very limited field settings far from areas of intended use, which presents certain unique environmental challenges. Among these are the following:

3.1 UV Resistance

Most fungal biocontrol agents are susceptible to UV damage, and high levels of exposure can greatly reduce the number of propagules that survive between the time of application and the time of exposure to the target (Leland and Behle 2005; Behle et al. 2006). The use of melanized microsclerotia provides some protection, but in many cases it is unclear if it is enough for the regions in which an agent would be deployed. New UV-tolerant strains might be required from areas subject to high levels of UV radiation. Isolation of such strains from areas whose climate approximates the environment could be carried out if needed, although direct isolation from the intended area of treatment is more desirable in order to obtain strains endemic (and therefore already adapted) to the area.

3.2 Environmental Variation

Environmental conditions and climate vary considerably between regions in which the same pest species might be found. Thus, it is possible that multiple formulations of a single biological control agent might be needed for a target pest species. For instance, consider populations of the mosquito *Aedes aegypti* on different continents. It is possible that a biopesticide formulated for use against populations in the temperate southeastern United States may not be effective for use in the highlands of western Kenya or the eastern jungle region of Peru. In such cases, the fungal agent would need to be re-formulated for use in those areas.

3.3 Economics and Local Implementation

In order for successful application of a biopesticide, the user must apply it correctly. This will be dictated by safety, ease of use, and affordability. In most developed nations, fungal entomopathogens are regulated by government agencies that evaluate their safety for human, animal, and environmental exposure and provide final certification and approval for their application in the field. While companies in developed nations are able to profit from the production of fungal biopesticides, this is not always the case for companies in developing nations. In such cases, alternative methods of production (such as growth media with sugar derived from local crops) would have to be developed so as to make the technology commercially viable and affordable to the local consumer. Moreover, the formulations to be developed would have to be in a form that could be easily used and applied by the average person. In many cases, manual broadcast, such as the use of granules that can be spread by hand, are favored because they are easy for anyone to apply as opposed to spray formulations that require more expensive equipment and, in some cases, refrigerated storage capabilities.

3.4 Acceptability by Local Communities

Regardless of the effectiveness of a biological control tactic, it will not succeed if it is not acceptable to the local communities. Thus, studies are required to assess the knowledge, attitude and perception of local communities on the use of biological control agents for pest control. Such studies must include an education component that informs members of local communities on the advantages of using such products as opposed to the use of toxic chemicals that might be easier to apply but more harmful humans, livestock and the environment. Outreach efforts might also be developed to include training members of local communities on how to use biological control products.

4 Plant Pests and the Need for Biocontrol Antagonists

With the increase in global trade, insects that vector plant diseases are being introduced with increasing frequency into new regions where they become invasive and threaten to wipe-out production of locally farmed crops. Management tactics usually first target the vectors and include mostly cultural control measures that involve destruction and removal of diseased plants. Efforts focusing on the deployment of biopesticides aim to use biocontrol agents that are effective in slowing the spread of the insect vector with the hope that the disease will subsequently be controlled. The problem, however, is that targeting the insect vector alone is not enough. In many cases, the pathogens are so virulent that only a single inoculation from the bite of one individual insect is enough to cause disease in the host plant; in such cases, the pathogen must also be targeted. Microorganisms that can be used to either suppress or eradicate a pathogen population are known as biocontrol antagonists.

Often the first step in the discovery process for new biocontrol antagonists is an in vitro screen for activity. This is not without controversy, as these screening methods have an inconsistent record of predicting future biocontrol efficacy (Lewis and Papavizas 1991; Campbell 1994), although this inconsistency likely results from the way in which screens are designed as opposed to the screening strategy itself. In that context, screens are usually designed to focus on one of three properties: (1) direct antibiosis, (2) ecological niche, or (3) amenability to the commercialization process. In many screens, all three properties are ultimately evaluated, either concurrently or sequentially. However, those that are absolutely required to assess antagonistic ability are based on direct antibiosis, for obvious reasons. Although it is important to consider the general morphology and growth characteristics of both the potential antagonists and the pathogen being screened against, Dickie and Bell (1995) performed a full factorial analysis of nine variables influencing in vitro antagonistic screens for potential biocontrol agents and identified the nine most important variables: (1) the strain of pathogen, (2) the strain of antagonist, (3) the growth media of the pathogen, (4) the growth medium of the antagonist, (5) the

temperature of growth of the pathogen, (6) the temperature of growth of the antagonist, (7) the pH of growth of the pathogen, (8) the pH of growth of the antagonist, and (9) the medium of the assay plate. Thus, an effective direct antibiosis screen will take into consideration all of these variables.

4.1 Direct Antibiosis Screening Assay

There are three assays that are most commonly used to conduct a direct antibiosis screen: (1) the seeded agar assay, (2) the agar overlay assay, and (3) dual point inoculations with growth assessment. Each assay type has its strengths and weaknesses associated with it. The seeded agar assay is a common assay to screen for biocontrol antagonists (Berg et al. 2000; Costa et al. 2006; Adesina et al. 2007). In these assays, the pathogen is typically incorporated into cooled melted agar (47–50 °C) and poured into plates. The potential antagonists are inoculated to the plates and the strains are allowed to grow for 2–3 days and at the end of the time period a zone of inhibition is measured. One of the primary advantages of using this type of assay is that the pathogen concentration is very consistent and uniform throughout the plate. This method is also useful for testing solutions (e.g. cell-free culture supernatant) for antibiosis activity. In the agar overlay assay, a plate with the media for the antagonist is inoculated and allowed to establish (Kelner 1948; Enefiok and Hagedorn 1978). Then a second agar medium is cooled (47–50 °C) and poured over the antagonist (Fig. 4.1). The pathogen is then plated on to the fresh surface. Alternatively, the pathogen could be incorporated into the overlaying media and introduced to the pathogen as a seeded agar. This assay format is primarily used when the antagonists grows with mycelial morphology (e.g. fungi or *Streptomyces* spp). If this assay is attempted with samples of a yeast-like morphology, it readily mixes with the second phase making the assay difficult to interpret. The agar overlay assay is particularly useful when the antagonist and pathogen display differences in preferred media composition. In the dual point inoculation assay, the potential antagonist is inoculated near the edge of the plate and allowed to establish (Fig. 4.2). The pathogen is then plated midway between the antagonist and the far side of the plate. After a set period of time, the distance from the pathogen inoculation point to the edge of the pathogen growth is measured on both the side with the

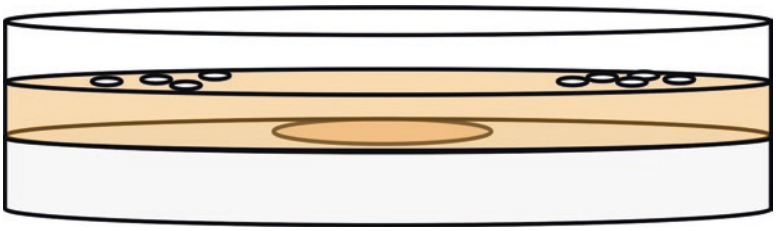

Fig. 4.1 Picture of an agar overlay assay

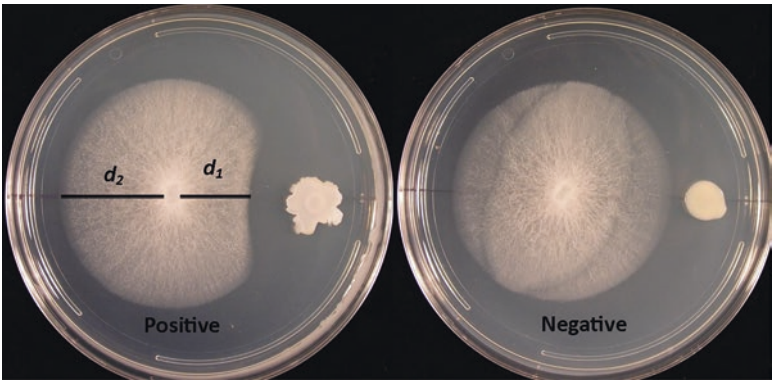

Fig. 4.2 A photo of a dual point inoculation assay with growth assessment; d_1 is the distance between the inoculation source and the growth toward the pathogen, d_2 is the distance between the inoculation source and the growth away from the pathogen

antagonist and the opposite side. The ratio of these to distances is the relative antagonism of the strain being screened (e.g. d_1/d_2 in Fig. 4.2). This allows for an easy ranking of the antagonists.

These three types of antibiosis assays all rely on the antimicrobial to be a diffusible molecule in the liquid of the agar plate. This means the active molecules can typically be found in the culture supernatant of a liquid culture of the antagonist. After a preliminary screening has detected some level of antibiosis antagonism, the cultures are usually grown in liquid media and the culture supernatant is evaluated for antimicrobial activity. In addition to the diffusible metabolites, it has been reported that volatiles can be an important class of secondary metabolites that can inhibit pathogens (Fernando et al. 2005). Recently, increased efforts have been applied to screening for antimicrobial volatiles from prospective biocontrol candidates (Aunsbjerg et al. 2015; Chaves-López et al. 2015; Hernández-León et al. 2015; Raza et al. 2015). These assays are typically conducted in one of two different formats. The first is using a divided plate assay, in which the different parts of a plate are physically separated from each other. The potential antagonist and the pathogen are grown on separate sections of the plate. A second method is to invert and place the plate of the pathogen over a plate of the antagonist, then seal the junction of the two plates to prevent the loss of volatiles. In addition to the considerations of the physical format of the antibiosis assay, there are other elements to consider. In some cases, in may be beneficial to amend the media of the assay to discourage or encourage specific modes of action. For example, iron availability can be limited to encourage siderophore mediated modes of action (Kloepper et al. 1980). Alternatively, the media can be amended with chitin or similar biopolymers to encourage the production of lytic enzymes, which have been shown to be a source of antifungal activity for biocontrol antagonists (Lim et al. 1991). In the case of very slow growing pathogens, it may be advantageous to use a proxy organism for the pathogen for a first screen (Pereira et al. 2013).

4.2 Identification and Biocontrol Potential of Antagonist Strains

Identifying strains that occupy the same ecological niche of a pathogen is another basis for screening strains as biocontrol antagonists. Strains that occupy the same ecological niche as the pathogen, can prevent the pathogen from become established. A few methods have been developed to screen organisms on the basis of ecological niche. One method relies are determining the number and type of substrates an organism can utilized. This is often accomplished with an automated phenotyping system (e.g., the Biolog® system). In such a system, the pathogen and pool of potential antagonists are screened for their ability to utilize a variety of carbon sources. The niche overlap index (NOI) is utilized as the basis of the screen; NOI is defined as the number of carbon sources utilized by each strain as a proportion of the total number of carbon sources utilized by the pair of strains (Wilson and Lindow 1994). NOI values >0.9 are considered to occupy the same resource niche, while NOI values <0.9 are considered to reside in separate resource niches (Wilson and Lindow 1994). Screening based on resource utilization does not always rely on screening a broad class of potential substrates. In some cases, the screen can target a specific resource believed to be important to the pathogen's establishment and infection. For example, Schisler et al. (2006) screened potential antagonists of *Fusarium* head blight based on their ability to utilize choline, which was thought to be an important pathogen signaling molecule. Beyond resource utilization, potential biocontrol antagonists are also screened for their ability to tolerate abiotic stresses anticipated in the field location. For instance, strains can be screened for their ability to grow at the same water activities and temperatures as the target pathogen (Janisiewicz 1996).

4.3 Industrial Forms

Amenability to the commercialization process is the final basis for which strains are screened. The aim of these screens is to evaluate how easily strains can be mass produced and whether or not they can be placed into a resting state (e.g., a spore) and reactivated, which is required for prolonged storage stability. Some microorganisms are capable of forming very stable resting forms, such as conidia, which make subsequent steps of the commercialization process easier. However, some non-spore-forming organisms, such as Gram-negative bacteria, are very difficult to prepare for long term storage stability (Segarra et al. 2015). Consequently, it is important to evaluate the candidate antagonists for their ability to survive the formulation process. In so doing, the screens typically use a microtiter plate format to evaluate growth under defined conditions (Köhl et al. 2011) and/or to evaluate drying of small samples of potential antagonists (Slininger and Schisler 2013), with assays designed to provide information on the number of colony forming units and propagule morphology in order to gauge growth, survivability, and activity.

5 Prospectus

The incorporation of living microbial biological control agents into integrated pest management programs is highly desirable because it reduces the use of chemical insecticides harmful to livestock, humans and the environment. In addition, it provides an alternative means to combat resistance to chemical insecticides. However, the commercial development of living microbial agents for insect pest control has lagged far behind in comparison to the development of chemical pesticides. Limited success in commercializing living microbial agents (especially fungi) is due in large part to the lack of effective strains to produce consistent pest control under field conditions. Therefore, researchers engaged in the development of biocontrol agents should also devote some of their work on finding superior biocontrol strains. One possible strategy in that regard is to genetically improve existing production strains, but this could eliminate the desired property of an agent being regarded as "natural". Being labelled a natural product is a primary advantage that biopesticides have over synthetic chemical pesticides in the arena of public perception, because of the prevailing attitude that synthetic chemicals are "bad" whereas natural products are "good". For that reason alone, oftentimes a consumer will elect to spend more money on a natural product in order to avoid buying a product that is synthetic or artificial. Thus, a better alternative for developing superior biocontrol strains is to conduct large scale screening studies of environmental samples. Moreover, instead of focusing attempts on finding new strains of known species by using selective protocols, new taxa could also be targeted by employing broad isolation methods. Unfortunately, there is a tendency in some circles to label such studies as fishing expeditions, although they need not be if care is taken to design them in a rationale way based on clear and focused objective that attack a well-defined question or hypothesis. Regardless, there is a clear need to develop new biocontrol strains for commercial application. With the increasing demand for agricultural products worldwide, especially as the global population increases, the need for sustainable agricultural practices will also grow; for that reason alone, microbial biocontrol agent research must also keep pace.

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Chapter 5
Fall Armyworm (FAW; Lepidoptera: Noctuidae): Moth Oviposition and Crop Protection

Camilo Ayra-Pardo and Orlando Borras-Hidalgo

If you know the enemy and know yourself, you need not fear the result of a hundred battles. If you know yourself but not the enemy, for every victory gained you will also suffer a defeat. If you know neither the enemy nor yourself, you will succumb in every battle.

Sun Tzu, "The Art of War"

Abstract In plant-herbivore insect interaction, moths have developed different odour-related behaviours to choose preferred hosts, evade plant defences and guarantee the offspring fitness. Fall armyworm (FAW) is a voracious polyphagous insect pest originally from the Western hemisphere that targets some of the most important crops Worldwide. Due to its aggressiveness as pest and high adaptability to adverse environments, the need for new pest control strategies is constant. The study of FAW behaviours to efficiently manage field populations and improve crop protection is of great interest to entomologists and plant scientists alike. This chapter presents an overview of fall armyworm as crop pest, highlighting some behavioural adaptations of ovipositing female moths that may undermine the effectiveness of existing crop protection practices if are not taken into account. Furthermore, this chapter addresses the recent use of novel ground-breaking gene-editing techniques such as CRISPR/Cas9 to functionally investigate the olfactory system in moths of *Spodoptera* sp. that will offer opportunities for the development of new pest control strategies.

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1 Introduction

'Good sense is as important as food'. This Irish proverb tells us about the important role sensorial system plays for living beings to make the right decision about what best food is. Sensory cues play a determinant role in many insect life situations e.g. food foraging, mating, oviposition. In lepidopteran insects, caterpillars eat voraciously to transform plant material into the tissues they will need to change into moths and produce the new progeny. The preference for a particular plant food is commonly associated with the adult oviposition choice (Jaenike 1978; Gripenberg et al. 2010). Therefore, the significance of a particular insect pest is given in the first place by the plant host chosen to oviposit by corresponding female moth.

Usually, female moths use plant odours i.e. semiochemicals to find the suitable host on which to oviposit for the fitness of the species. Moths prefer normal than stressed plants since the latter accumulate poor-quality nutrients and antifeedant metabolites that would eventually reduce offspring performance. During evolution, plants have acquired an arsenal of defensive responses for deterring herbivores that includes low molecular-weight metabolites (so-called secondary metabolites) and proteins that exert toxic or antinutritional effects e.g. wound-inducible proteinase inhibitors (Howe and Jander 2008; Zhu-Salzman et al. 2008). To counteract, insects have co-evolved to produce effectors that suppress plant defences. For instance, the application of insect oral secretion (OS) from *Manduca sexta* to *Nicotiana attenuata* leaves reduced wound-induced expression of plant-defence threonine deaminase and nicotine accumulation (Kahl et al. 2000; Schittko et al. 2001). Glucose oxidase present in saliva of several caterpillars inhibits nicotine production in tobacco (Musser et al. 2005). OS of the Colorado potato beetle *Leptinotarsa decemlineata* diminishes wound-induced accumulation of proteinase inhibitor transcripts in tomato (Lawrence et al. 2007). Plants have also indirect defence responses to feeding larvae such as the herbivore-induced plant volatiles (HIPV), which is considered a 'call for help' to attract predators and parasitoids i.e. natural enemies of herbivores, which can exert a control on pest populations (van Poecke and Dicke 2004). Indeed, the exploitation of natural enemies for pest control has become very popular in integrated pest management (IPM) programs (Barzman et al. 2015; Lamichhane et al. 2017), and combined with other practices e.g., pheromone trapping, habitat management (control of alternative hosts), mechanical 'pupae busting', can be effective to bring down pest population sizes.

The fall armyworm (FAW) *Spodoptera frugiperda* S. (Lepidoptera: Noctuidae) is native from tropical regions of Western hemisphere where is considered a pest since it prefers to feed on economically important cultivated grasses such as maize, rice, sorghum, sugarcane but also other vegetable crops and cotton (Sparks 1979). Historically, extensive applications of chemical insecticides have been used to control this pest but this practice has proved to be neither economically nor environmentally sustainable and could present health risks. Besides, FAW has shown a great capacity of building resistance against synthetic pesticides (Yu 1991; Carvalho et al. 2013). The use of genetic engineering to make plants produce its own FAW-specific and safe insecticides, like genetically-modified (GM) plants expressing

Bacillus thuringiensis (Bt) insecticidal proteins, has been a viable alternative for maize and cotton crops so far (James 2016). Instead, ecologists, environmentalists and the United Nations' Food and Agricultural Organization (FAO), recommend an integral management for this pest, following a sustainable IPM approach. This includes a deep knowledge of the insect, its life stages, biology, natural enemies and aspects of behavioural ecology in order to get a better implementation of control programs. For example, the impact of FAW female moth behaviours like the oviposition preference in response to induced and constitutive plant volatiles for the effectiveness of current crop protection practices need to be revisited.

2 FAW: Geographical Distribution of a Reknown Agricultural Pest

The FAW is a moth belonging to the superfamily Noctuoidea. It is a voracious generalist insect that feeds during its larval stage over 100 plant species, including economically important crops. The literature on this insect as agricultural pest is extensive (Sparks 1979; Todd and Poole 1980; Whitford et al. 1988; Ashley et al. 1989; Lewter et al. 2006). Since its wide host range, FAW is one of the most harmful pests threatening annual crops in the Western hemisphere.

In the southeastern United States, Central America and the Caribbean, FAW is considered the most important pest of maize causing millions of dollars in damage to farmers every year and food shortages in poorest populations (Painter 1955; Ortega 1987; de Lange et al. 2014). FAW insects prefer immature plants as food; accordingly, mature larvae can cause severe damages to crop yields depending on defoliation levels (Fig. 5.1). In 2016, FAW was reported for the first time in Africa, where it was found destroying maize crops in twenty-one countries in the south and west parts of the continent (BBC 2017). Since FAW is considered a strong flier, capable of long-distance dispersal in warmer climates, it is currently considered a serious threat to the European continent after the global temperature raises due to climate change.

3 FAW: Life Cycle

Like all lepidopteran insects, FAW undergoes complete or holometabolous metamorphosis. The life cycle includes four developmental stages that is egg, larvae, pupae and adults, each with its own morphology (Fig. 5.2). The entire cycle is completed in approximately 31–55 days depending on the ambient temperature. The number of generations occurring in an area varies with the appearance of the dispersing adults. The ability to diapause is not present in this species, so it can be only found the entire year in tropical regions, where it usually occurs as a polyvoltinous species with overlapping generations.

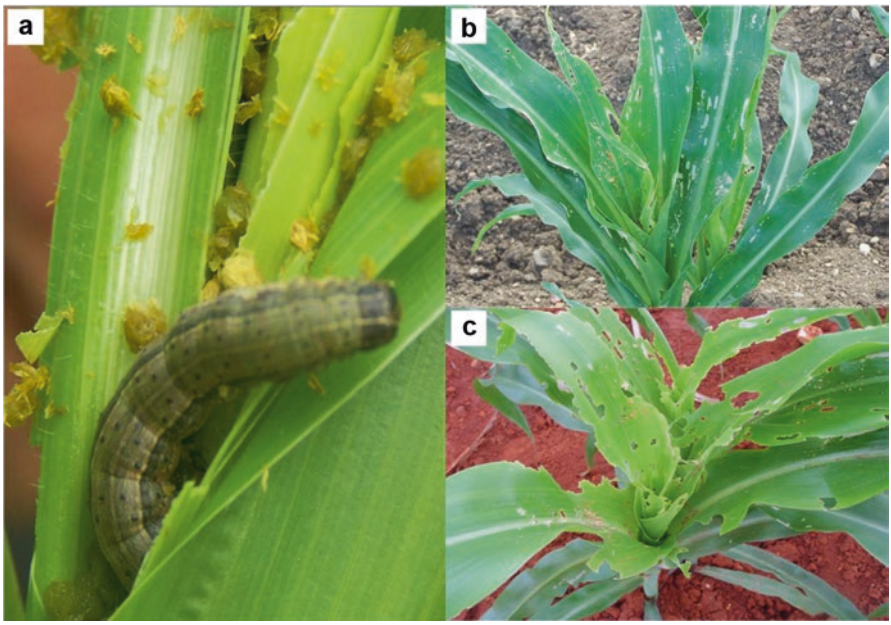

Fig. 5.1 Type of lessons caused on maize plants by the fall armyworm, *Spodoptera frugiperda* Smith. (**a**) Fall armyworm larva feeding on a maize plant. (**b**) Minimal visible leaf damage, with several small to mid-sized elongated lesions on a few whorl and furl leaves. (**c**) Extensive leaf damage, with whorl and furl leaves of the plant almost totally destroyed

Egg The egg is dome shaped, with a flat base. The number of eggs per mass is often about 100–200, and total egg production per female averages about 1500. The eggs are deposited in layers. SEM analysis has shown FAW egg masses are firmly attached to the plant surface with excretions from female accessory glands that function as glue (Peñaflor et al. 2011). A layer of grayish scales is deposited over the egg mass by ovipositing female adult that gives a furry or mouldy appearance.

Larvae There usually are six instars in fall armyworm. Instars are visibly differentiated by the width of head capsule, ranging about 0.3–2.0 mm from instars 1–6. In addition, larval length can reach 34.2 mm at the instar 6. The larval colour varies according to the food diet. A distinctive feature of this species is the existence of a white inverted "Y" in the head of mature larva and a rough or granular texture epidermis. Duration of the larval stage tends to be about 14 days during the summer and 30 days during cold weather. Mean development time was determined to be 3.3, 1.7, 1.5, 1.5, 2.0, and 3.7 days for instars 1–6, respectively, when larvae were reared at 25°C (Pitre and Hogg 1983). Larvae are very active in foraging; following hatching, they disperse from the original plant to search for another host by crawling and ballooning. Olfactory studies have shown this caterpillar is more attracted by herbivore-damaged than undamaged maize plants (Peñaflor and Bento 2011). According to these authors, the volatile linalool produced at higher doses by dam-

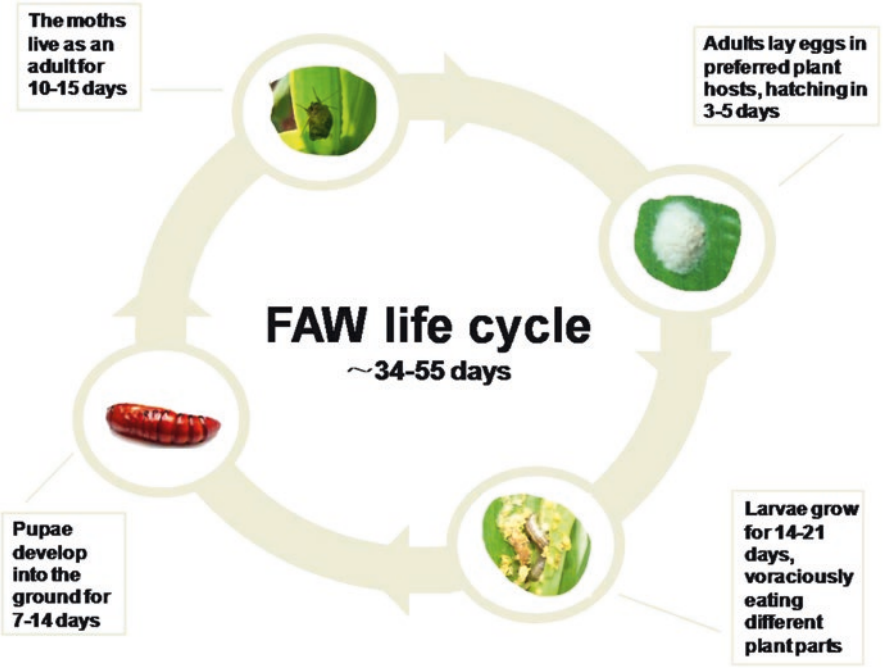

Fig. 5.2 Fall armyworm life cycle. Fall armyworm undergoes complete metamorphosis in which it goes through four different life stages that is egg, larva, pupa and adult. The insect starts its life as an egg. Then, neonate larvae hatch from eggs and start eating plant leaves almost constantly. Larvae molt six times as they grow. Finally, larvae turn into pupae where profound morphologic and physiological changes take into account before emerging as flying adult moths. Adults will reproduce to start again the cycle. The time length of each stage depends on environmental and physiological factors

aged than undamaged maize plants is apparently the responsible for larva attraction. The explanation could be larvae feel attracted by odours from damaged plants because they are more detectable and thus they are able to quickly find their host and not crawl for long distances.

Pupae Pupation normally takes place in the soil, at a depth 2–8 cm. The pupae are reddish brown in color, and measure 14–18 mm in length and about 4.5 mm in width. The pupal stage of fall armyworm cannot survive prolonged periods of cold weather (Pitre and Hogg 1983).

Adult The moths have a wingspan of 32–40 mm. In the male moth, the forewing generally is shaded gray and brown, with triangular white spots at the tip and near the center of the wing. The forewings of females are less distinctly marked, ranging from a uniform grayish brown to a fine mottling of gray and brown. The hind wing is iridescent silver-white with a narrow dark border in both sexes. Adults are nocturnal and most active during warm, humid evenings. They can perceive volatile plant

secondary metabolites i.e. semiochemicals by a highly sensitive olfactory system that depends on olfactory receptor neurones (ORNs) in sensible, mostly on the insect antennae, the main olfactory organs (Bruce et al. 2005; Hansson and Stensmyr 2011; See Chaps. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2). Within sensible, odour recognition relies on the expression of a diversity of olfactory genes located on the cell membranes of olfactory receptor neurons (Legeai et al. 2011). Since plant host selection is crucial for offspring performance, mated female moths use the olfactory system to discriminate between suitable and unsuitable plants as hosts.

4 FAW: Host Crop Plant Preference

In FAW, two morphologically identical sympatric host strains distinguished by genetic markers have been recognized (Levy et al. 2002; Nagoshi and Meagher 2003; Nagoshi et al. 2006a). They are commonly referred as the rice-strain (R-strain) and the corn-strain (C-strain) according to their distribution among plant hosts in field surveys (Nagoshi and Meagher 2004a, b), with C-strain larvae preferentially found on maize (*Zea mays* L.), sorghum (*Sorghum* spp.), and cotton (*Gossypium hirsutum* L.) plants, and R-strain larvae associated with rice (*Oryza sativa* L.) and forage grasses i.e. *Cynodon* spp. and other species (Pashley et al. 1985, 1987; Pashley 1986; Nagoshi et al. 2007). The two strains were initially detected when host-associated electrophoretic differences were observed at five allozyme loci (Pashley 1986). Other strain-specific genetic markers have since been detected (Lu et al. 1992, 1994; Nagoshi 2010).

The association of each strain with a particular plant is apparently not absolute. For instance, individuals showing the C-strain haplotype have been found in pasture habitats where R-strain population is predominant, and vice versa, larvae with R-strain markers have been isolated from maize or cotton fields where the largest presence for C-strain is usually present (Prowell et al. 2004; Nagoshi et al. 2006b, 2007; Machado et al. 2008). The reasons for this variability are not known yet, but could reflect plasticity in strain behaviour or be associated with the occurrence of inter-strain hybrids. In fact, pheromone trapping routinely attract males of both strains, indicating a chance for inter-strain mating (Meagher and Nagoshi 2004; Nagoshi and Meagher 2004b). Nevertheless, the asymmetric distribution of the two strains with selective plant host preference is consistently observed.

Efforts have been made to determine the basis for FAW strain-specific differences in plant host distribution. Experiments on larval survival on different plant hosts have shown both strains are capable of efficiently utilizing the preferred host plant of the other indicating that differential larval feeding is unlikely to be sufficient to explain observations of host fidelity in the field (Groot et al. 2010). On the contrary, strain-related differences in the host preference for oviposition have been detected under laboratory conditions (Hay-Roe et al. 2011).

Host-preference has a strong component of the olfactory sensory system (see Chaps. 1, 2 and 3, volume 2). The involvement of olfactory genes such as those

controlling odorant-binding proteins (OBPs) and chemosensory proteins (CSPs, see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2) has been previously linked with host plant specialization processes in fruit flies (Matsuo et al. 2007; McBride 2007; Linz et al. 2013; Ramasamy et al. 2016; Comeault et al. 2017), mosquitoes (McBride et al. 2014) and aphids (Smadja et al. 2012; Duvaux et al. 2015; Eyres et al. 2016). OBPs and CSPs are small soluble proteins highly expressed in the odor sensory organs of insects (see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2). They have been largely described for their ability to interact with distinct hydrophobic odorant and pheromone molecules. They would facilitate their transport to olfactory receptors (ORs, see Chap. 4, volume 2), thereby initiating the signal transduction process in specific sensory neuronal cells (Xu et al. 2005; Hallem et al. 2006; Sánchez-Gracia et al. 2009). These properties suggest that host specialization would likely involve evolution at olfactory genes (see Chap. 10, volume 2).

Host choice can also be a source for pre-mating isolation between sympatric populations (Via et al. 2000), and host specialization has been previously linked with assortative mating processes in Lepidoptera (Emelianov et al. 2001, 2003; Malausa et al. 2005). In many insect species, mating behaviour is modulated by sex pheromones and olfactory sensory components have a strong influence in the process. In most of the cases, females release long distance pheromones to attract potential mates (Cardé and Baker 1984) while male sex pheromones act at short distances during the final phase of courtship (Royer and McNeil 1992, 1993; Picimbon 1996). Therefore, the pheromone production and responsiveness to the each other 'chemical' call signal are crucial aspects for a successful mating, which could be explored for environmentally-safe biological control of pest species, such as moths (see Chaps. 11 and 12, volume 2).

In FAW, the Rice-strain and Corn-strain have shown a limited inter-strain mating in both laboratory and field studies (Pashley and Martin 1987; Whitford et al. 1988; Pashley et al. 1992) that have suggested they may actually be cryptic species. The analysis of major pheromone components in gland extracts of both strains found significant differences in the concentration and relative proportions of the different compounds as a function of physiological parameters such as female age (Lima and McNeil 2009). However, the differences were in the same magnitude of intra-specific variability reported in other Lepidoptera species, so this aspect alone is not sufficient to ensure reproductive isolation of the two strains.

5 Pest Management

5.1 Chemical Insecticides

The use of chemical pesticides to control FAW infestations is a frequent practice when the defoliation is noticed in the plant crop (Pitre and Hogg 1983; King and Saunders 1984; Carvalho et al. 2013). In maize crop, larvae prefer to feed on early vegetative stages of the plant rather than reproductive stage. Usually, solitary larvae

can be found feeding deep in the whorl of young maize plants covered with a heavy layer of frass. This behaviour prevents insecticides penetration, so a high volume of sprayed products are required to obtain adequate control. The problem is that repeated exposures to chemical pesticides of multiple generations can select for resistant FAW strains (see Chap. 3). So far, a broad spectrum of resistance has been reported based on multiple mechanisms acting separately or in concert, including increased detoxication by microsomal oxidases and target site insensitivity (Yu et al. 2003).

5.2 *Insect Pathogens*

Numerous pathogens have been associated with FAW but only a few cause epizootics (Gardner and Fuxa 1980; Gardner et al. 1984). The *S. frugiperda* nuclear polyhedrosis virus (NPV) is among the most important causing high levels of mortality in some populations (Cruz et al. 1997). However, disease typically appears too late to alleviate high levels of plant defoliation.

Another entomopathogenic agent used in biological control of FAW is the *B. thuringiensis* bacterium (Bt; see Chap. 4). This microorganism acts in the insect gut, where bacterium spore-accompanying crystals solubilise into protoxins that are converted in toxic polypetides (delta-endotoxins) by gut proteases. The activated toxins join to specific receptors in the apical membrane of midgut columnar cells and induce cell death by a mechanism not totally understood (Sanahuja et al. 2011; Adang et al. 2014). The massive destruction of larval midgut epithelium ultimately causes insect death by inanition and septicaemia. Most typical Bt formulations consist of spore/crystal preparations obtained from cultures in fermenters; the preparations are dried and used as wettable powders, spray concentrates and liquid concentrates (Ifoulis and Savopoulou-Soultani 2004; Brar et al. 2006; Singh et al. 2010). Since current Bt strains tend not to be very potent against FAW and persistence of Bt formulations in the field is still low, the use of this kind of product as alternative control method is often questionable. However, the isolation of new strains with higher insecticidal activity for FAW and new technological developments in the pipeline of bioproducts engineering is expected to change the panorama in the nearest future (also see Chap. 4).

5.3 *Natural Enemies*

The use and exploitation of natural enemies to control insect populations is common in agro-ecological practices. Numerous species of parasitoids and predators affecting fall armyworm have been recorded (Rios-Velasco et al. 2011); however, the dominant species often varies from place to place and from year to year (Fig. 5.3). Luginbill (1928) and Vickery (1929) were pioneers in the study of fall armyworm

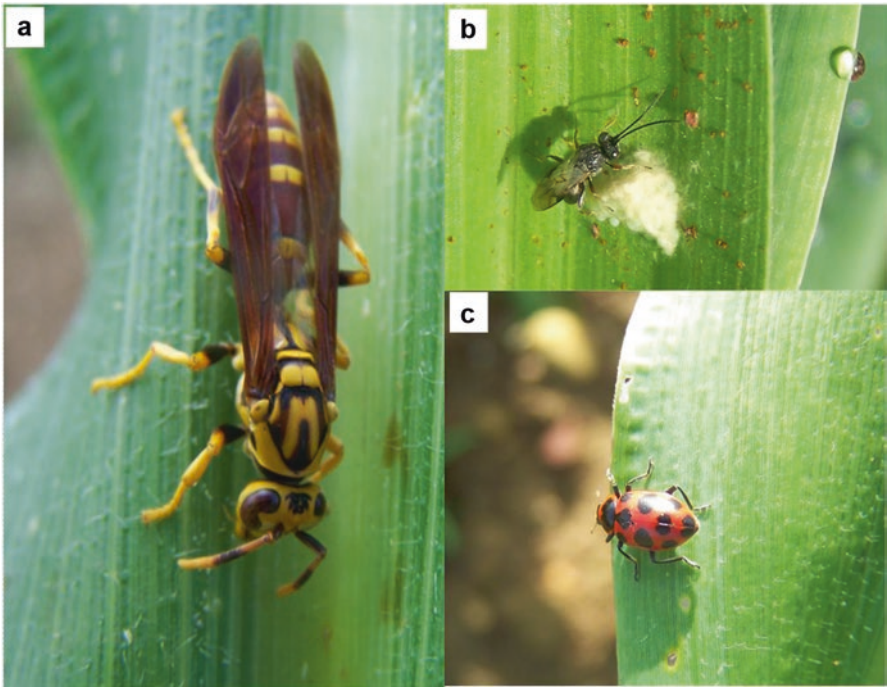

Fig. 5.3 FAW natural enemies from a maize crop field in Cuba. (**a**) Predator wasp *Polistes cubensis* Lepeletier (Hymenoptera). (**b**) Parasitic wasp *Telenomus heliothidis* Ashmead (Hymenoptera). (**c**) Predator beetle ladybird *Coleomegilla cubensis* Csy. (Coleoptera)

parasitoids. Since chemical insecticides can harm arthropod parasitoids of herbivore insects, a reduction in pesticides use is a key element when these organisms are incorporated to control programs. Instead, natural enemies can be better attracted and conserved using intercrop-based strategies of pest suppression like the so-called 'push-pull' strategy (Cook et al. 2007). This strategy is based on the employment of plants that emit volatiles that can either repel (push) insect pests from the crop or attract (pull) them into trap crops, thereby impacting insect behaviour. Therefore, these practices employ and exploit traditional farming strategies based on plant chemistry, which may also be ecologically justified and sustainable.

5.4 Bt Crops

Since 1996, plants have been modified to express genes coding for Bt insecticidal toxins (Sanahuja et al. 2011). This way, plants themselves produce the insecticidal factor that protects themselves from insect pests without the need for any external sprays. In 2016, 25.2 million hectares of Bt crops, mainly corn and cotton, were grown globally (James 2016). Among the multiple benefits Bt crops offer,

a reduction in the environmental impacts from pesticides and the increased opportunity for beneficial insects – Bt proteins do not kill beneficial insects – are the most relevant. The major concern for Bt-crops use has been always resistance evolution in key pest species (Tabashnik and Carrière 2017). Fundamental to resistance management is the 'high dose/refuge' strategy (Huang et al. 2011). High doses should effectively kill heterozygous carriers of resistance alleles; homozygous susceptible pests emerging from a conventional (toxin-free) refuge should constitute the bulk of the insect population. Mating between male and females heterozygous carriers should therefore be extremely rare, ensuring that homozygous resistant individuals with effective phenotypic resistance will be even rarer. This strategy is considerably improved with the use of crops expressing multiple toxin genes to provide redundant killing (pyramid strategy), which can only be overcome by pests possessing multiple independent resistance mechanisms (Tabashnik and Carrière 2017). Effective conventional refuge area is also essential for these multi- toxin crops. Despite all this, reports on control failures in the field increased from 3 cases in 2005 to 16 cases in 2016 (Tabashnik and Carrière 2017). Three of these refer to FAW resistance against Bt maize event TC1507, expressing the Bt Cry1Fa toxin, in Puerto Rico (Storer et al. 2010), Brazil (Farias et al. 2014) and the southeastern United States (Huang et al. 2014).

5.5 Integrated Pest Management (IPM)

IPM, also known as integrated pest control (IPC), is a broad-based approach that integrates different practices for economic control of pests. IPM aims to suppress pest populations below the economic injury level. The UN's Food and Agriculture Organisation defines IPM as "*the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms*".

An integrated approach for FAW control can been facilitated by combining cultivation practices with varieties carrying insect-resistant traits either by natural or GM means, the use of biological controls including natural enemies, and a reduced spraying of insecticides. The most employed cultural practice is early planting and/or early maturing varieties (Mitchell 1978). Also, early harvest protects maize ears from FAW generations that develop later in the season.

The use of parental lines from locally-adapted germplasm, with an herbivory-resistant phenotype, is a plus in sustainable crop breeding programs. A good example in maize breeding is the inbred line Mp708, highly accepted as parental line since its proved efficacy to withstand herbivory by the FAW and other lepidopteran caterpillars (Brooks et al. 2007). Quantitative trait loci (QTL) mapping have shown

that several genes known to confer resistance to several lepidopteran insects and related pests have been successfully bred into Mp708 (Brooks et al. 2007). Thus, Mp708 possesses typical maize fitness traits and the additional trait of maintaining readiness for resisting insect attack. It appears to achieve this, in part, by constitutive expression of the maize insect resistance-1-cysteine protease (Mir1-CP), whose locus lies in one of the resistance QTL to FAW (Jiang et al. 1995; Brooks et al. 2007). Once ingested, the proteolytic activity of Mir1-CP damages the insect's peritrophic matrix, impairing nutrient utilization (Chang et al. 1999; Pechan et al. 2002, 2004; Mohan et al. 2006, 2008).

6 FAW-Plant Interaction

Jasmonic acid (JA), an important phytohormone that is synthesized by an oxylipin pathway, plays a central role in plant inducible defences against herbivores. In fact, herbivore damage elicits a rapid and transient JA burst in the wounded leaves and JA functions as a signal to mediate the accumulation of various secondary metabolites that confer resistance to herbivores (Verhage et al. 2010). Accordingly, *Nicotiana attenuata* plants impaired in jasmonate production or perception clearly show that herbivore-induced defences are largely controlled by JA signalling (Halitschke et al. 2003; Paschold et al. 2007).

In FAW-maize plant interaction, a model for the regulation and induction of maize *mir1* gene, encoding the Mir1-CP protein involved in the defence against herbivore, was proposed to use the JA pathway (Ankala et al. 2009). These authors suggested a signalling pathway initiated by an herbivore elicitor that stimulates the JA pathway and transduces different responses downstream via the Ethylene (ET) phythormone. ET regulates both *mir1* transcript expression and Mir1-CP accumulation in a two-branched constitutively maintained active pathway that guarantee relatively high basal levels of *mir1* transcript and a rapid induction and accumulation of Mir1-CP.

Insect eggs have also shown to trigger a plant defensive response. The production of ovicidal substances in rice in response to eggs of the white-backed plant hopper (Seino et al. 1996), the development of necrotic zone at the site of egg deposition in *Brassica nigra* and potato to cause egg mortality (Balbyshev and Lorenzen 1997), and the emission of plant volatiles (mono- and sesquiterpenes) resulting in attraction of egg parasitoids (Meiners and Hilker 2000; Hilker and Meiners 2002; Fatouros et al. 2005) are all examples of direct and indirect responses of host plants to moth oviposition.

Interestingly, the expression profile of *Arabidopsis* leaves has been found to be altered after oviposition by *Pieris brassicae* moths (Little et al. 2007). Egg deposition induced in situ the expression of hundreds of *Arabidopsis* genes, including defence and stress-related genes, and stimulated callose deposition, hydrogen peroxide production and cell death. Egg-derived elicitors of unknown nature have been suggested to be responsible for the plant response to moth oviposition. Interestingly,

some of the oviposition-induced genes are known targets of salicylic acid (SA). This signalling molecule is a potent inducer of pathogenesis-related genes, and is involved in the resistance against biotrophic pathogens (Glazebrook 2005). It is well known SA and JA signalling pathways interact antagonistically (Beckers and Spoel 2006; Koornneef and Pieterse 2008). In fact, several studies have shown SA can suppress JA-dependent defence responses (Doares et al. 1995; Gupta et al. 2000; Spoel et al. 2003, 2007; Cipollini et al. 2004; Koornneef and Pieterse 2008), and hence, weakens the plant response to attackers combated through the JA pathway.

Oviposition by *S. frugiperda* in maize plants suppressed the emission of several constitutive and herbivore-induced volatiles (Peñaflor et al. 2011). Accordingly, ovipositing female moths may have evolved the ability to indirectly manipulate plant direct and indirect defence signalling, through as yet unknown egg elicitors, in order to change the type of defensive response and hence guarantee the offspring fitness.

7 FAW Damage-Avoiding Oviposition and Crop Protection

The reason many insects avoid laying eggs on herbivore-damaged plants is presumably an adaptive response, selected to minimize intra-specific competition and predators/parasitoids attack (De Moraes et al. 2001; Harmon et al. 2003). Besides being a 'call for help' for natural enemies of herbivore insects, HIPVs can also mediate interactions with ovipositing female moths (Halitschke et al. 2008) that perceive plant odours through the highly sensitive chemosensory receptor neurones of the insect antennae, in their way to locate suitable plants as hosts and to avoid unsuitable hosts (Bruce et al. 2005).

In 1970, Schurr and Holdaway used a laboratory olfactometer to investigate the *Ostrinia nubilalis* (European corn borer; Lepidoptera: Pyralidae) moths response to volatiles from their host plant. The study revealed for the first time that volatiles from injured host plants have a deterrent effect on moths. Later, Renwick and Radke (1982) found that larval damage from *Trichoplusia ni* (Cabbage looper; Lepidoptera: Noctuidae) to a host cabbage plant produced volatiles i.e. HIPV that significantly repealed conspecific females. In tobacco, some HIPV compounds were released exclusively at night by plants damaged from *Heliothis virescens* (Tobacco budworm; Lepidoptera: Noctuidae) larvae and proved to be highly repellent to conspecific female moths during behavioural assays (De Moraes et al. 2001). In FAW, damage-avoiding oviposition behaviour of female moths was initially reported by Signoretti et al. (2012). The olfactory response of FAW-mated female moths toward odours released by undamaged and herbivore-induced maize plants showed a strong preference for the former. The potential impact of this behaviour on resistance evolution to the Bt toxins present in Bt crops, was not revised until very recently.

Bt supplies the vast majority of insecticidal compounds for today's transgenic crops (James 2016). This is a safe, effective technology with proven environmental

benefits relative to synthetic chemical pesticides (Carrière et al. 2003; Christou et al. 2006). Preserving this technology depends upon managing the evolution of resistance in the insect pests consuming these crops. Considerable research effort has been invested in modelling and studying the evolution of resistance to Bt transgenic crops (Brevault et al. 2013). In the 'high dose/refuge' strategy, the high doses ensure that resistance is effectively recessive, while non-Bt refuge are planted near Bt crops so that any emerging homozygous resistant adults will tend to mate with susceptible partners and so produce heterozygous, phenotypically susceptible progeny (Gould 1998; Tabashnik et al. 2008; Huang et al. 2011). Current resistance management assumes that oviposition is random across Bt crops and non-Bt refuge.

Recently, a higher and consistent oviposition preference for Bt over non-Bt maize plants was described in FAW female moths, during six independent experiments in 3 years (Tellez-Rodriguez et al. 2014). Moreover, in this study, the preference for Bt maize plants was correlated with the degree of feeding damage in non-Bt maize plants (refuge), indicating that females preferred not to lay eggs on damaged plants. Then, Tellez-Rodriguez et al. explored the impact of non-random oviposition on the evolution of resistance to Bt toxins under diverse management scenarios using simulation models. The simulation model was parameterized using field observations of oviposition behaviour and survivorship from this study, and on published data on the genetics of resistance. The model was built on an established population genetics and population dynamics framework (Raymond et al. 2007). The results demonstrated that this behaviour could undermine the efficacy of the 'high dose/refuge' strategy (Fig. 5.4). Besides, the nature of density dependent mortality in non-Bt refuge could moderate or exacerbate the influence of non-random oviposition.

There is enormous international interest from policy makers, governments and scientists in managing resistance to Bt crops effectively. Also, there are increasingly frequent reports of rising resistance to Bt crops in some countries (Tabashnik et al. 2013; Tabashnik and Carrière 2017). The results from Tellez-Rodriguez et al. (2014) are likely to be of wide significance in agriculture industry because they probably apply to a large number of key agricultural pests, such as moths. We speculate that this kind of behavioural factors might be far more important in the tropics where pests are polyvoltinous or have overlapping generations and the damage caused by preceding pest generations could have big effects on resistance management. High levels of FAW resistance to the same Bt maize in Puerto Rico (Storer et al. 2010), Brazil (Farias et al. 2014) and the southeastern United States (Huang et al. 2014) led to severe crop losses. The results from Tellez-Rodriguez et al. (2014) suggest that oviposition bias could have help circumvent this problem. Since damage levels are linked to pest population sizes, and damage levels determine the efficacy of resistance management, the modelling results in FAW demonstrate that pest population dynamics are far more important for resistance management than previously believed.

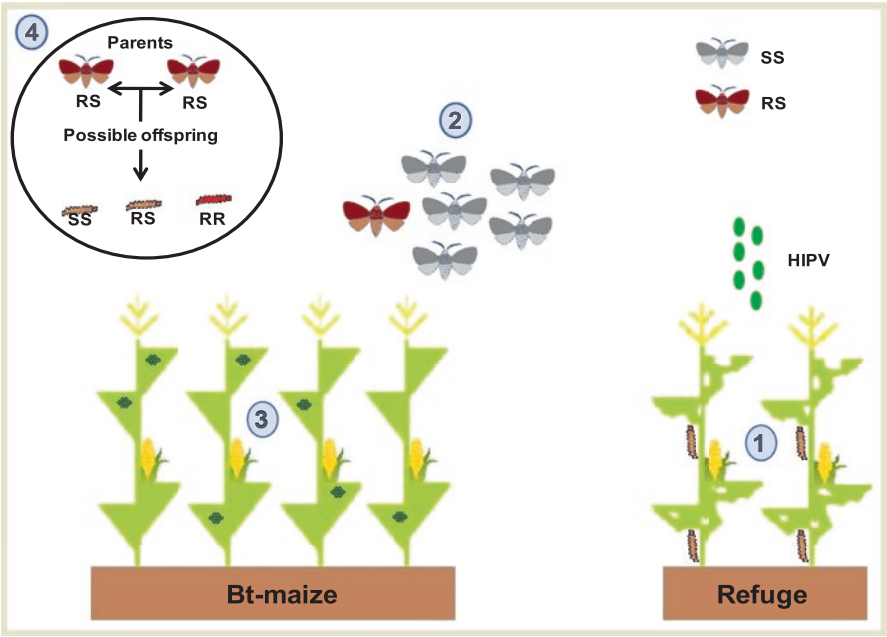

Fig. 5.4 Damage-avoiding oviposition behavior and Bt-resistance evolution. (*1*) FAW larvae freely feed on conventional maize plants of the refuge while 'high dose' Bt maize plants would kill newly hatched susceptible (SS) and the 99.99% of the heterozygote (RS) larvae soon after emergence. (*2*) As the growing season progresses, the HIPV released during larval feeding from the refuge plants can deter oviposition in female moths. (*3*) A strong oviposition preference for Bt over non-Bt refuge plants in FAW moth is manifested. (*4*) The few RS moths that could emerge from the Bt-crop field (0.01%) would mate each other producing homozygous (RR) resistant individuals in the progeny

8 Toward a Functional Study of the Moth Olfactory System Through Epigenetics

Genetic mutants are critical for the study of gene functions. In the past, mutants have been randomly obtained by physical, chemical, or biological (e.g. transposon insertion) means to unravel the molecular basis of different biological mechanisms. However, random mutagenesis often produce unexpected effects, and farther large-scale mutants screening is tedious and costly (McCallum et al. 2000). In recent years, sequence-specific nucleases (SSNs), including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 (CRISPR/Cas9) have been demonstrated to be useful tools for genome editing in many organisms, including insects (Huang et al. 2016). Compared with the first two other genome editing technologies, CRISPR/Cas9 remains substantially less expensive and much easier to program for editing new target sites.

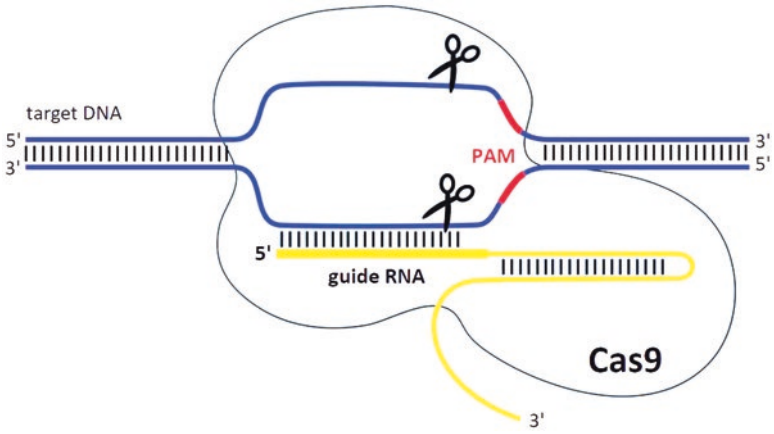

Fig. 5.5 CRISPR/Cas9 mechanism. The essential step in editing an organism's genome is selective targeting of a specific sequence of DNA. Two biological players, the Cas9 protein and guide RNA, interact to form a complex that could identify target sequences with high selectivity. The Cas9 protein is responsible for locating and cleaving target DNA, both in natural and in artificial CRISPR/Cas systems

The CRISPR/Cas9 technology, a prokaryotic defence system originally identified in *Streptococcus pyogenes*, typically utilize two components, the CRISPR-associated protein 9 (Cas9) and a single guide RNA (sgRNA), to perform genome-editing and other molecular functions (Golic 2013). In principle, the complex formed by Cas9 and sgRNA can be targeted to any specific genomic sequences to create DNA double-strand breaks (DSB), which are subsequently repaired primarily by the high-fidelity homologous recombination (HR) or error-prone non-homologous end joining (NHEJ) pathways. The NHEJ often introduces small insertion or deletion (In-Del) mutations at the cut site that lead to the loss of gene function (Fig. 5.5).

Since the discovery of the CRISPR/Cas9 system, scientists have exploited properties of this DNA cleavage mechanism to allow directed alterations to be made within the genomes of virtually any organism. So far, CRISPR/Cas9 has been successfully used in several insect species for exploring the gene function (*Aedes*: Basu et al. 2015; Kistler et al. 2015; *Bombyx*: Wang et al. 2013; Liu et al. 2014; *Drosophila*: Bassett and Liu 2014; *Tribolium*: Gilles et al. 2015). Compared with RNA interference (RNAi) technology, which works on the transcription level, the CRISPR/Cas9 system works on the genome level and is more penetrating and useful for functional genes analysis than RNAi in many cases. Besides, RNAi technology does not provide an efficient means for blocking lepidopteran genes (Terenius et al. 2011).

Recently, the first three studies describing successful application of CRISPR/Cas9 mutagenesis in two *Spodoptera* pest species i.e. *S. litura* and *S. littoralis*, were published (Bi et al. 2016; Koutroumpa et al. 2016; Zhu et al. 2016). Interestingly, two of these studies investigated the potential application of CRISPR tech in functional study of hypothetical genes as being involved in insect olfaction. In Zhu et al.

(2016), the *SlitPBP3* gene from *S. litura* encoding the pheromone binding protein 3 was the target (see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2). PBP3 is supposed to be a rather ancient molecule transporter, the result of a gene duplication that has occurred before the split of moth into various species (Picimbon and Gadenne 2002; Picimbon 2003; Abraham et al. 2005). In the oriental leafworm moth, Zhu et al. reported 87% of chimeras in surviving injected embryos and adults. Homozygous *SlitPBP3* knockout mutants were tested for their response to three main sex pheromone components using electro-antennography technique (EAG; see Chaps. 1, 2, and 3, volume 2). The antennal responses persisted in PBP3-mutants, suggesting a minor role for this protein in pheromone perception.

In the second study, Koutroumpa et al. (2016) performed mutagenesis on the *Orco* gene of *S. littoralis*. In *D. melanogaster*, ORCO forms heterodimers with olfactory receptors (ORs) that act as odor-gated ion channels at the membrane of olfactory sensory neurons (Smart et al. 2008; Wicher et al. 2008; see Chap. 4, volume 2). Therefore, Orco represents a good target gene to investigate olfactory pathways in insects, particularly in agricultural pests such as Lepidoptera. Compared with Zhu et al. (2016), this new study found 89.6% of the injected individuals carried Orco mutations and 70% of those transmitted them to the next generation. *Orco* knockout mutants were assessed regarding the ability of the antennae to respond to a variety of plant volatiles as well as sex pheromone components. Homozygous ORCO-mutant moths were shown to be anosmic not only to plant odors but also to sex pheromonal compounds, similarly to mutant mosquitoes that showed loss of olfactory acuity after targeted mutations in the orco gene (DeGennaro et al. 2013).

These congruent studies in mosquitoes and moths give two examples of molecular evidence that can be used to help determine the sensory acuity of a target insect. Similarly to flies, mosquitoes and other biting Diptera, herbivorous species such as Lepidoptera suffer critical lack of genetic tools and heritable genome edition systems like CRISPR/Cas 9 very likely can open new perspectives not only for functional genomics studies, but also for new opportunities in applied science. The particular study of ORCO by CRISPR techniques in mosquitoes and moths demonstrates the efficiency of targeting specific olfactory genes to lead to the development of new strategies alternatives to insecticide and/or microbial sprays for insect pest control.

9 Concluding Remarks

Insects were among the earliest terrestrial herbivores and acted as major selection agents on plants. Plants evolved chemical defences against these herbivores and the insects, in turn, evolved mechanisms and behaviours to deal with plant defences. In our relationship with insects, some classify as crop pests due to their preference for the same plants we use for food (monophagy). Some species like FAW even target more than one crop plant (polyphagy) and have shown a great capacity to a rapid adaptation to synthetic insecticides. Therefore, an integral approach that goes beyond chemical control is necessary to efficiently manage insect pest populations

in crop fields. The knowledge of insect behavioural ecology is an essential part of this strategy. Together with the development of genetic and gene knock-out technology such as CRISPR, the Identification of pheromones and host plant odor volatiles combined with our understanding of insect chemosensorial systems will certainly bring hope and new elements for the development of more ecologically-justified and sustainable methods of control in this "struggle" between human, environment protection and pest species in the twenty-first century.

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Chapter 6
Olfaction as a Target for Control of Honeybee Parasite Mite *Varroa destructor*

Victoria Soroker, Nitin Kumar Singh, Nurit Eliash, and Erika Plettner

Abstract The mite *Varroa destructor* Anderson & Trueman (Acari: Varroidae) is a major global threat to the European honeybee *Apis mellifera*. The mite is an obligatory ectoparasite. It feeds on the hemolymph of bees and also serves as an active vector for pathogenic viruses, which have become more abundant and virulent since the invasion of the mite. The Varroa life cycle is tightly linked to that of a honeybee. The cycle can be generally divided into two main phases: a reproductive phase, in which the female Varroa parasitizes bee pupae and reproduce within sealed brood cells, and a phoretic phase, in which it parasitizes adult bees. Between these phases Varroa mites can wander on comb surfaces. Hive volatiles, mainly from adult bees and brood, play a crucial role in the parasite's life cycle, by guiding host finding, selection and regulating its reproduction suggesting that the mite's olfaction may be an important target for new specific control agents. This concept was proven with some synthetic volatile compounds. Inhibition of host sensing leads to incorrect Varroa host selection or reduction in mite's ability to reach a host. Although the mode of action of these compounds is not yet clear, this approach seems promising towards an integrated and sustainable control over this major apicultural pest.

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1 Introduction

The European honeybee *Apis mellifera* is a major pollinator of agricultural crops and as such is of high importance to food production and agricultural security (Watson and Stallins 2016). The mite *Varroa destructor* Anderson & Trueman (Acari: Mesostigmata: Varroidae) is a most important parasite and major global threat to the European honeybee almost worldwide (Nazzi and Le Conte 2016). This rather big mite (1.5 mm wide), originally a parasite of *A. cerana* in Asia, extended its host range to *A. mellifera*, when the latter was introduced to Asia for honey production. The mite has been described both from *A. cerana* (original host) and *A. mellifera* (new host), formerly erroneously also classified as *V. jacobsoni* (Rosenkranz et al. 2010). To date about 60–100 years after the host switch, two almost clonal types of *V. destructor* from Korea and Japan/Thailand have been detected in *A. mellifera* colonies (Beaurepaire et al. 2015). It is unknown to parasitize any other insect species (Sammataro and Avitabile 2011).

Varroa is an obligatory parasite that spends its entire life in a honeybee colony, feeding on the hemolymph of the adults or pupae. The parasitism by the mite weakens the bee's immune system and makes it more vulnerable to other secondary pathogens, shortens the bee's life span, decreases its weight, the lifetime flight duration and non associative learning abilities (Nazzi and Le Conte 2016). Varroa mites also serve as an active vector of pathogenic viruses, which have become more abundant and virulent since the invasion of the mite (Genersch 2010; De Miranda et al. 2011; Zioni et al. 2011). Approximately 20 honeybee viruses have been discovered and the majority of them have an association with Varroa mites (De Miranda et al. 2011).

Varroa life cycle is intimately linked to that of a honeybee. It can be generally divided into two main phases: a reproductive phase, in which the Varroa parasitizes honeybee pupae and reproduces within sealed brood cells, and a phoretic phase, in which the female Varroa parasitizes an adult bee (Rosenkranz et al. 2010; Nazzi and Le Conte 2016). Between these phases the female mites move freely on the surface of the comb (Eliash et al. 2014). The male's life is constrained to the capped brood where it mates with its sisters. Its lifespan is limited to the duration of honeybee pupation period and thus usually varies from 10 (in worker brood) to 12 days (in male brood). Males do not survive outside the cell. On the other hand, female mites may have up to three reproductive cycles. The reproductive cycle starts after the female mite enters into the brood cell. It invades the cell in the prepupal stage of the brood before capping, 20 h and 45 h for worker and drone brood, respectively (Boot et al. 1993), and starts oogenesis within a few hours (Rosenkranz and Garrido 2004). The female mite lays the first male egg approximately 70 h after cell capping followed by 3–5 female eggs in 30 h intervals (Rehm and Ritter 1989; Martin 1994).

Emerging Varroa female may be phoretic on any adult bee but rarely on a queen. During the phoretic phase, Varroa has two avenues for spreading: on nurse bees, Varroa can reach the brood area, and on forager bees mites spread to other colonies during robbing or drifting (Goodwin et al. 2006). The phoretic stage lasts about 5–11 days when there is brood in the colony. In the absence of honeybee brood, as

occurs during non-reproductive periods of the honeybee colony, mites remain phoretic for extended periods (Rosenkranz et al. 2010).

If infested colonies remain untreated they are expected to die within 2–3 years, while already in the 1st year their productivity and/or pollination performance will be impaired (Currie and Gatien 2006; Guzman-Novoa et al. 2010). Selection of appropriate treatment within the hive is a big challenge in terms of selectivity (efficacy to the parasite but low toxicity to the bee) and residual activity. In particular, the fact that honeybee products, both hydrophilic (honey and royal jelly) and hydrophobic (wax and propolis) are used as food, medicines or in cosmetics, requires special concern. Various chemicals and biotechnical methods have been used to reduce mite load; most noted is the implementation of synthetic acaricides such as an organophosphate, coumaphos, the pyrethroids tau-fluvalinate and flumetrin, as well as a formamide, amitraz. Despite their negative effects they are easy to implement, making them popular among beekeepers. However, as could be predicted, Varroa populations are developing resistance to these synthetic acaricides, rendering them ineffective in many areas, e.g. in Israel in 2018 amitraz started to lose its efficacy, after mites already gained resistance to both tau-fluvalinate and coumaphos. The most commonly used soft acaricides are organic acids (formic and oxalic) or the monoterpene thymol from essential oil (Rosenkranz et al. 2010; Plettner et al. 2017). The applications of the latter are less favored by professional beekeepers, due to its inconsistent efficacy and side effects on the bees. Other well-described possibilities to reduce Varroa infestation are biotechnical methods such as: the "trapping comb technique" and "brood removal", both are labor intensive and not widely accepted. In light of an immense need to develop new specific and environmentally sustainable ways for Varroa management, a chemo-ecological approach was suggested already by Yoder and Sammataro in 2003 and recently by Plettner et al. (2017). This chapter focuses reviewing Varroa host chemosensing and its potential disruption.

2 Varroa-Honeybee Chemical Communication

In the dark hive environment and in the absence of eyes for the mites, chemical cues play an essential role in the tight association between the mite's and bee's life cycles. Chemical cues are important for the mite's host finding, selection as well as initiation of reproduction (Rosenkranz and Garrido 2004; Pernal et al. 2005; Cabrera Cordon et al. 2013). Its phoretic host preference is highly adaptive. At low Varroa load in the colony, mites have been shown to prefer nurse bees over older (foragers) and even more so, over young newly emerged bees (Kraus 1994; Xie et al. 2016). However, this preference is context dependent, as it changes under conditions of high infestation or when colonies are close to collapse. At these conditions, nurses and foragers are similarly attractive to mites (Cervo et al. 2014). This shift is highly adaptive enabling mites to escape a "sinking ship" but at the same time not losing any opportunity to reach the reproductive host as long as it exists. This modulation

of Varroa preference is apparently regulated by the input from the hive environment. Varroa is known to respond to a number of chemical cues released from the bees and its environment (Fig. 6.1). The activity of particular compounds was assessed by laboratory behavioral bioassays. In many cases, the method implemented to determine the activity of the tested compounds was ambiguous in discriminating between a true attraction and arrestment of a wandering mite. We thus classified the compounds according to the results as reported (Table 6.1).

Varroa discrimination between nurse and forager bees is most likely based on both high and low volatility compounds, such as: (a) a lower titer of the Nasonov gland pheromone component geraniol (which strongly repels the mite) in nurses than in foragers, (Hoppe and Ritter 1988) or (b) nurse or forager specific blends of cuticular hydrocarbons (Del Piccolo et al. 2010). The difference in hydrocarbon profiles was found to be obscured under high infestation level (Cervo et al. 2014).

It has been noticed that Varroa mites prefer drone brood over worker brood, likely because the titer of fatty acid esters secreted by the drone brood is higher than that produced by worker (Le Conte et al. 1989; Trouiller et al. 1992; Sammataro et al. 2000). The first step of the activation of the mite's oogenesis is triggered by volatiles of the larval cuticle (Rosenkranz and Garrido 2004). The activating com-

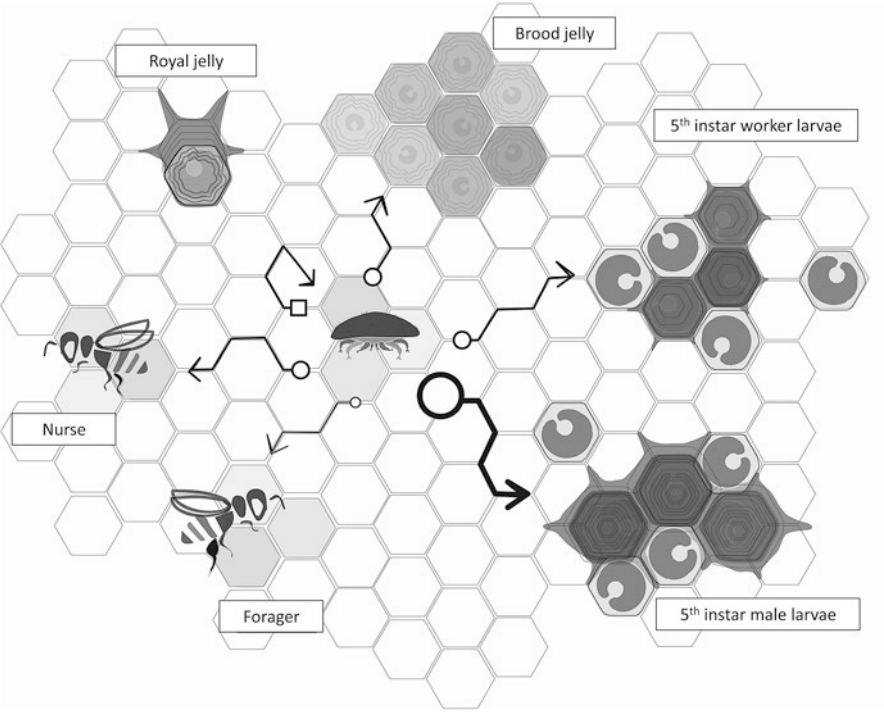


Fig. 6.1 Chemical cues from various hive sources affecting Varroa movement. Arrows directed to the odour source indicate attractants/arrestants, arrows diverted from the source indicate repellents. (Original drawing by Dan Eliash)

Table 6.1 Hive chemical cues and their effect on Varroa

Chemical	Source	Effect on Varroa	References	Effect on bees	References
Geraniol	Adult bee -Nasonov gland, mainly foragers'	Repellency	Hoppe and Ritter (1988) and Pernal et al. (2005)	Orientation, attractive to foragers	Pickett et al. (1980) and Slessor et al. (2005)
Nerolic acid	Adult bee -Nasonov gland, mainly foragers'	Repellency	Pernal et al. (2005)	Orientation, attractive to foragers	Pickett et al. (1980) and Slessor et al. (2005)
1-octanol	Adult bee – Koschevnikov gland	Repellency	Kraus (1990)	Recruiting, alarm pheromone	Slessor et al. (2005)
Octanoic acid	Brood jelly mainly Royal jelly	Repellency	Nazzi et al. (2009)	NR	
Aliphatic alcohols and aldehydes, Eicosane	Bee pre-pupa cuticle	Arrestment	Donzé et al. (1998)	NR	
2 – heptanone	Bee mandibular glands	Paralysis and death	Papachristoforou et al. (2012)	Repels foragers	Breed et al. (2004)
2 – hydroxyhexanoic acid	Brood jelly	Attraction	Nazzi et al. (2004)	NR	
Hydrocarbons					
C_{19}–C_{29} hydrocarbon chains	5th instar bee larva- cuticle	Attraction	Rickli (1992)	NR	
C_{17}–C_{22} hydrocarbon chains	5th instar bee larva- cuticle	Arrestment	Calderone and Lin (2001)	NR	
(*Z*)-8-heptadecene	1. Infested cells[a]	1. Reproduction	1. Milani et al. (2004)	NR	
	2. Foragers' cuticle	2. Repellency	2. Del Piccolo et al. (2010)		
Fatty acid methyl esters					
Methyl palmitate	5th instar bee larva- cuticle	1. Attraction[b]	1. Le Conte et al. (1989)	Brood pheromone	Le Conte et al. (1990) and Alaux et al. (2009)
		2. Oogenesis[c]	2. Frey et al. (2013)		
Methyl stearate	5th instar bee larva -cuticle	1. Oogenesis	1. Frey et al. (2013)		

(continued)

Table 6.1 (continued)

Chemical	Source	Effect on Varroa	References	Effect on bees	References
Methyl linoleate	5th instar bee larva -cuticle	1. Attraction	1. Le Conte et al. (1989)		
		2. Oogenesis	2. Frey et al. (2013)		
Methyl linolenate	5th instar bee larva -cuticle	1. Attraction	1. Le Conte et al. (1989)		
		2. Oogenesis	2. Frey et al. (2013)		
Methyl oleate	5th instar bee larva -cuticle	1. Oogenesis	1. Frey et al. (2013)		
Fatty acid ethyl esters					
Ethyl palmitate	1. Freshly molted female Varroa-cuticle	1. Eliciting male mating behavior	1. Ziegelmann et al. (2013)	Brood pheromone, Queen retinue pheromone	Le Conte et al. (1990), Slessor et al. (2005), and Alaux et al. (2009)
	2. 5th instar bee larva- cuticle	2. Attraction	2. Le Conte et al. (1989)		
		3. Arrestment	3. Donzé et al. (1998) and Calderone and Lin (2001)		
		4. Oogenesis	4. Frey et al. (2013)		
Ethyl stearate	1. Freshly molted female Varroa-cuticle	1. Eliciting male mating behavior	1. Ziegelmann et al. (2013)	Queen retinue pheromone, primer pheromone	Slessor et al. (2005) and Keeling et al. (2003)
	2. 5th instar larva cuticle	2. Oogenesis	2. Frey et al. (2013)		
Ethyl oleate	1. Freshly molted female Varroa-cuticle	1. Eliciting male mating behavior	1. Ziegelmann et al. (2013)	Brood pheromone, Queen retinue pheromone, primer pheromone	Leoncini et al. (2004), Alaux et al. (2009), and Castillo et al. (2012)
	2. Fifth instar bee larva cuticle	2. Attraction	2. Le Conte et al. (1989)		
	3. Bee's crop	3. Oogenesis	3. Frey et al. (2013)		
Palmitic acid	1. 5th instar bee larva- cuticle	1. Attraction[b]	1. Rickli (1992)	Brood pheromone	Le Conte et al. (1990)
	2. Freshly molted female Varroa-cuticle	2. Eliciting male mating behavior	2. Ziegelmann et al. (2013)		

NR Effect on bees not reported

[a]Infested cells – Nazzi and Milani (1996) have found that the extract of artificial-gelatin-cell containing infested larva have reduced the number of the mite's offspring. It is unclear if the origin of the chemical is from the bee or the mite (Nazzi et al. 2002)

[b]Attraction – Conflicting results, showing no attraction (Donzé et al. 1998; Boot 1994; Rickli et al. 1994)

[c]Oogenesis – The pupa cuticle emits two groups of compounds: Ethyl and Methyl esters. Changes in relative ratio of the two groups along the development of the pupa indicate female Varroa suitability of the host for reproduction. Details are presented in the text

ponents are apparently in the polar fraction of the brood cuticular volatiles (Trouiller and Milani 1999; Rosenkranz and Garrido 2004). It has been also reported that a reasonable percentage of female mites do not reproduce successfully after invading a brood cell. Some mites do not lay eggs at all (reviewed in Corrêa-Marques et al. 2003; Rosenkranz and Garrido 2004; Carneiro et al. 2007; Rosenkranz et al. 2010), others do lay male or female eggs only or show delayed egg laying (Donzé et al. 1994; Martin et al. 1997; Locke 2015). The reasons for these disorders in mite reproduction are still unknown. It can partially result from some problems in host sensing.

2.1 Structure and Function of the Varroa Pit Organ

Not much is known concerning structure and function of olfactory organs of mites in general and of Varroa in particular. Mites lack antennae, however their front legs (forelegs) appear to act as such, as mites hardly can be seen using them for walking, rather, they are seen lifting and waving them above the surface sampling the air (Fig. 6.2).

In the forelegs, the Varroa olfactory organ consists of a sensory pit that resembles the structure of Haller's organ found in ticks (Axtell et al. 1971; Allan 2010). Scanning electron microscope (SEM) show that the sensory pit organ of Varroa consists of nine internal sensilla (S1–S9) and nine long hair sensilla surrounding the organ (R1–R9, Fig. 6.3; Milani and Nannelli 1988; Dillier et al. 2006). Some of the sensilla (at least six) are wall-pore sensilla, similar in their external-morphology to the olfactory sensilla of other Arthropods (Fig. 6.4). The olfactory hairs in ticks as in insects are hollow structures, into which the dendrite(s) of the olfactory neurons project. These dendrites are surrounded by a fluid, the sensillar lymph (Tichy and Barth 1992). The pit organ is also present in the male Varroa forelegs, and was shown to be crucial for the initiation of copulation behavior by the adult male (Häußermann et al. 2015).

In addition to the forelegs, the mite bears chemosensory sensilla on other parts of the body, in particular on the pair of chelicerae and pedipalps that form the mite's mouthparts, like in other Arachnida (De Bruyne and Guerin 1998; Hebets and Chapman 2000). Evidence for chemosensilla was found on the female Varroa pedipalps (Liu and Peng 1990) and other mouth parts (Nuzzaci et al. 1992). However their function in host sensing is yet unclear.

2.2 Function of Varroa Pit Organ

The electrophysiological assay known as the ElectroAntennoGram (EAG) is the most common measure to test the electrophysiological response of insect antennae to volatiles and for identification of blends' active components, presented in a solvent on a filter paper or as an output of GC or GCMS (GC-EAD) (Bjostad 2000).

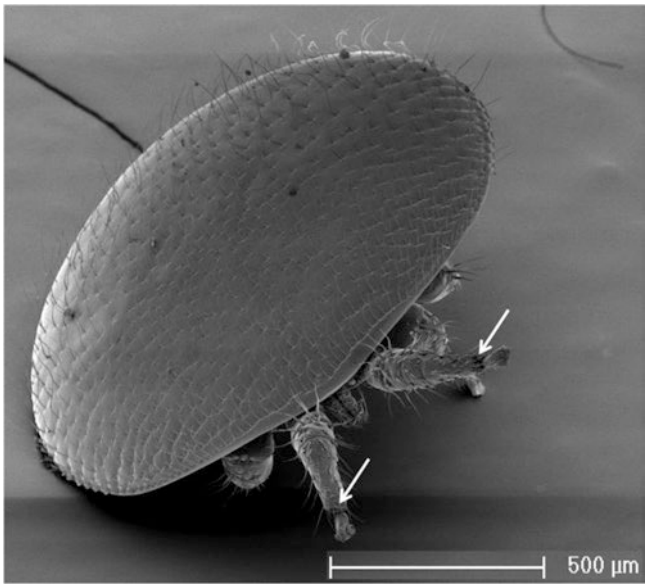


Fig. 6.2 Scanning electron microscope, anterior view at an adult female *Varroa* mite. Arrows show the location of the pit organ at the external distal part of the mite's foreleg. Bar = 500 µm

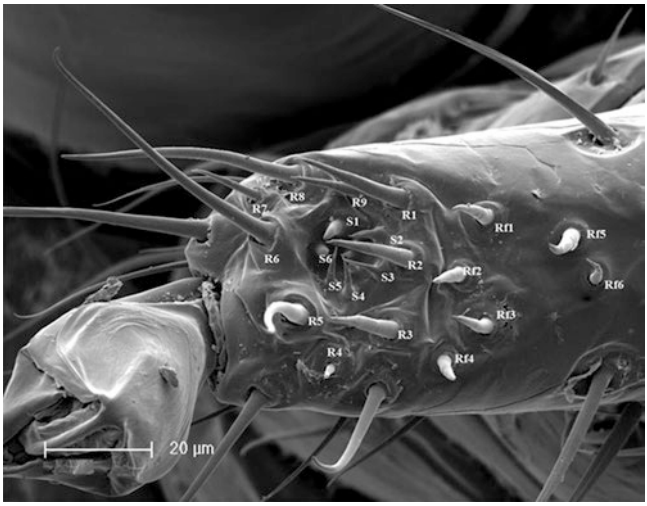


Fig. 6.3 Scanning electron microscope of the pit organ on the mite's foreleg tarsi. Sensila inside the pit marked by S1–S6; Sensila serounding the pit marked by R1–R9. Bar = 20 µm

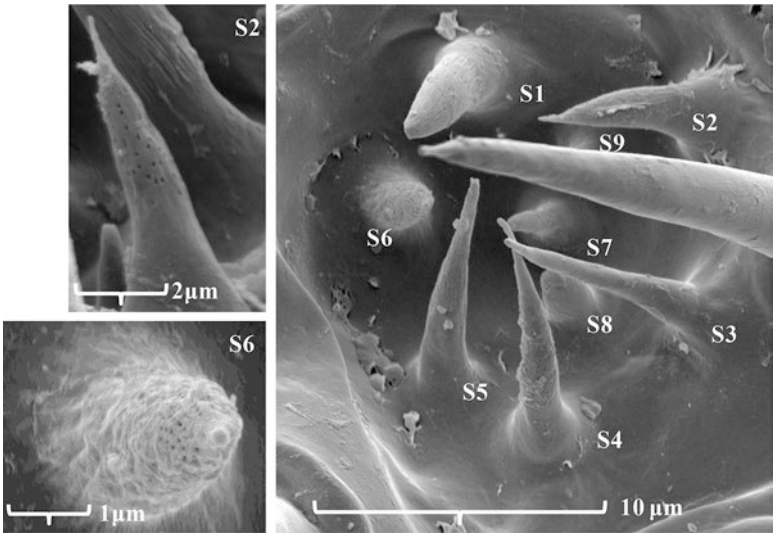
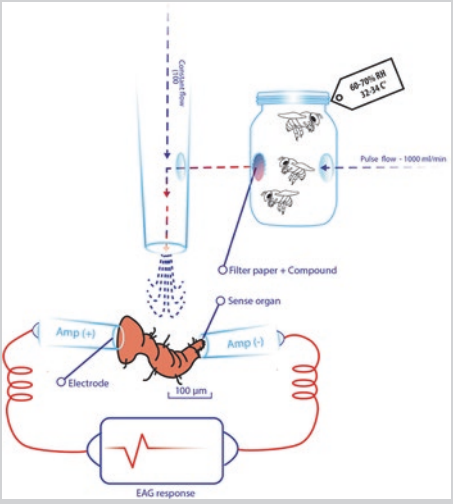


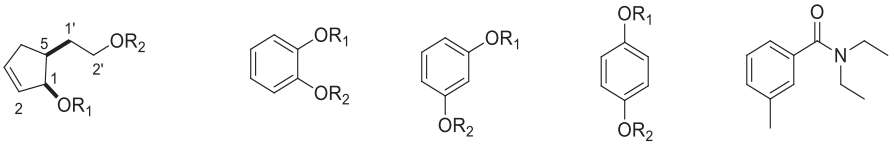
Fig. 6.4 Scanning electron microscope image of the pit organ (on the right) and the chemosensila in it (on the left)

In case of Arachnids, since the recording is conducted from the foreleg, ElectroTarsoGram (ETG) is a more appropriate term. A few recordings from Arachnids' forelegs were documented so far (whip spider *Phrynus parvulus* (Hebets and Chapman 2000) and cattle tick, *Boophilus microplus* (De Bruyne and Guerin 1998). ETG recordings from Varroa clearly showed that Varroa is sensing chemical cues by its foreleg (Endris and Baker 1993; Dillier et al. 2001; Eliash et al. 2014), particularly bee odors (Box 6.1; Fig. 6.5). So far Varroa foreleg was found to respond in ETG to both nurse and forager honeybee volatiles as well as to honeybee pheromone of the honeybee colony, E-β-ocimene (Eliash et al. 2014; Singh et al. 2014, 2016).

Translation of chemical cues into an electrical signal that activates the nervous system is presumably stereotypic, generally requiring four stages: odorant solubilization/transport through aqueous lymph; recognition by olfactory receptors on the dendritic membrane; activation of ion channels; and signal removal/degradation. Although the genomes of various taxa have been already partially sequenced, our current knowledge of Arachnid's olfaction is still heavily based on data acquired from insects and in particularly on the vinegar fly, *Drosophila melanogaster*. These have led to the identification of some major components of the olfactory machinery, in particular soluble odorant-binding proteins (OBPs), membrane-bound olfactory receptors (ORs) and a co-receptor (Orco), associated proteins such as sensory neuron membrane proteins (SNMPs), ionotropic receptors (IRs), and odorant-degrading enzymes (ODEs) (Leal 2013; see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2).

Box 6.1
Electrophysiological activity of the isolated foreleg was measured by means of Electrotarsogram (ETG) recordings. The foreleg of a female Varroa was cut at the base and placed between two glass microcapillaries containing silver electrodes and conductive saline KCl 0.1N. A constant flow of charcoal-filtered and humidified air was blown towards the leg. The leg was then stimulated by honey bee had space by puffing air (0.5 s, 1000 ml/s) through a frozen killed bee/s, or a similar size empty glass jar as a control. The receptors were allowed to recover for 0.5–2 min between stimuli. This setup was successfully used for evaluation of olfactory disruptive compounds (Eliash et al. 2014; Singh et al. 2015).





Alkyl chain codes: 1 = methyl, 2 = ethyl, 3 = propyl, 4 = n-butyl, 5= n-pentyl

Fig. 6.5 Structures of the compounds tested for disruption of Varroa host sensing

Despite the general similarity in olfactory machinery, a one-size-fits-all mechanism of olfaction is inconsistent with arthropod semiochemicals diversity and chemosensory specificity. So far, not much is known concerning the mechanism behind odorant detection in Arachnids in general and Varroa in particular. As for molecular

components of the olfactory machinery, members of both soluble carrier proteins and chemoreceptors were reported in a recent transcriptomic, proteomic and genomic studies on Arachnids. The studies have revealed some communalities with known insects' chemosensory proteins, along with a few either divergent or additional families (see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2). The classic insect-OBPs as well as the OR chemoreceptor family are absent from Arachnids as from all non-insect Arthropods investigated so far (Peñalva-Arana et al. 2009; Vieira and Rozas 2011; Chipman et al. 2014; Pelosi et al. 2014; Gulia-Nuss et al. 2016; Hoy et al. 2016; Vizueta et al. 2017; Eliash et al. 2017; Renthal et al. 2017; Iovinella et al. 2018; see Chaps. 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2). On the other hand, genes and/or proteins of GRs and IRs (the chemoreceptor subfamily of IGRs; see Chap. 4, volume 2), were identified in almost all reported non-insect Arthropods (Peñalva-Arana et al. 2009; Croset et al. 2010; Chipman et al. 2014; Gulia-Nuss et al. 2016; Hoy et al. 2016; Ngoc et al. 2016; Vizueta et al. 2017; Eliash et al. 2017; Eliash et al. 2019; Iovinella et al. 2018), except for *Amblyomma americanum* (Renthal et al. 2017). Among the latter, orthologs of one specific IR, IR25a, conserved across protostomes, was found in *Varroa destructor* as well as in other Arachnids: *Ixodes scapularis, Dysdera silvatica and Tetranychus urticae* (Missbach et al. 2014; Ngoc et al. 2016; Vizueta et al. 2017; Eliash et al. 2017). SNMPs are membrane bound proteins found to play a role in insect chemosensing, by mediating pheromone detection in association with chemoreceptors (Vogt et al. 2009; Gomez-Diaz et al. 2016). In Arachnids, transcripts with the conserved domain of "CD36 family" were found so far in the spider *D. sylvatica* and in Varroa (Vizueta et al. 2017; Eliash et al. 2017). However, their expression profile suggests that their function in chemosensation is less likely (Eliash et al. 2019). Recently, the diverse families of Transient Receptor Potential (TRPs) and degenerin/Epithelial Na+ Channels (ENaCs) were suggested to have an additional role as chemoreceptors in insects, and were reported to be expressed in Varroa (Peng et al. 2015; Eliash et al. 2019) as well in *Tetranychus urticae* genome (Ngoc et al. 2016). Regarding soluble carrier proteins, though chemosensory protein (CSPs) were proposed before to be the main soluble carrier proteins in non-insects, the presence of CSPs in Arachnids is not consistent (Vieira and Rozas 2011; Iovinella et al. 2016; Renthal et al. 2017; Eliash et al. 2017; see Chaps. 6, 9 and 10, volume 2), and its role in chemosensation seems questionable (Xuan et al. 2015). Considering the vague function of CSP is Arachnids' chemosensation and in the absence of insect-OBPs, two other potential odorant carriers were recently proposed: Niemann-Pick disease protein, type C2 (NPC2) and OBP-like proteins (see Chaps. 7 and 8, volume 2). NPC2 is a subfamily of the lipid carriers-Lipocalins (see Chap. 7, volume 2). Recently, NPC2 homologs were detected in Arachnids (Pelosi et al. 2014; Iovinella et al. 2016; Vizueta et al. 2017; Renthal et al. 2017) including Varroa (Eliash et al. 2017). In addition, potential proteins with a few features similar to insect-OBPs, including a six-cysteine profile, were recently found in the tarsi of the first pair of legs of *A. americanum* (Renthal et al. 2017). Later on, similar transcripts were found based on sequence similarity in Varroa mite (Vizueta et al. 2017; Eliash et al. 2017; Iovinella et al. 2018).

Nonetheless, all the data on non-insect potential chemosensory genes and proteins, is focusing on sequence similarities and phylogenetic analysis, while only one functional work was published on Varroa and *T. mercedesae* TRPA1, showing activa-

tion of the L isoform of the channel by electrophilic compounds and plant derived chemicals, such as α-Terpineol, that also repels Varroa in laboratory and hive experiments (Peng et al. 2015; Dong et al. 2016). The possible function of the above mentioned potential olfactory/chemosensory proteins (for NPC2 and OBP homologs; see Chaps. 6, 7, 8, 9, and 10, volume 2) remains to be proven in the Varroa mite. Recently, we have identified putative odorant receptor transcription factor (PRTF) in Varroa. Silencing this gene using RNAi, results in simultaneous down-regulation of Varroa host sensing behavior leading to decrease in the mite's ability to reach a host (Singh et al. 2016). Exactly which genes are regulated by PRTF-like is still a mystery.

3 Disruption of Varroa Host Association

The idea to use chemical cues to control Varroa is not a new one. Several publications raised the possibility to trap Varroa by using various honeybee volatiles that were reported to attract Varroa (Rosenkranz et al. 2010). However, this approach did not prove to be effective so far. On the other hand, the importance of chemical cues in parasite-host association is obviously one of the Varroa mite's Achilles's heel. Once host chemosensing is disrupted it can be expected that proper host selection and/or reproduction will be significantly impaired.

Despite much progress in the identification of host olfactory cues guiding Varroa, neither effective attractants nor repellents have been found so far. In view of limited success in exploiting hive semiochemicals in Varroa control, we evaluated synthetic disruptive compounds that were originally developed in the Plettner laboratory for the disruption of sexual communication in Lepidoptera (Plettner and Gries 2010) (see Chap. 11, volume 2) and commercial mosquito repellent N,N-Diethyl-meta-toluamide (DEET) (Singh et al. 2015; Pinnelli et al. 2016).

3.1 Structure and Function of Chemosensory Disruptive Compounds

Over 14 chemicals from three classes of compounds: dialkoxybenzenes, ethers of 5(2′-hydroxyethyl) cyclopent-2-en-1-ol and DEET were evaluated in a stepwise manner. The first step was screening by ETG (Eliash et al. 2014). The foreleg was stimulated by puffs of honeybee odor, or a clean air (control) as shown in Box 6.1. Once a foreleg preparation was found responsive to a positive bee volatile (head space of five nurse bees) the tested chemicals were blown on the leg with or without positive stimulus (nurse headspace). In particular, we have compared the effect of four dialkoxybenzenes that showed activity against insects (Akhtar et al. 2007, 2010; Plettner and Gries 2010), and six *cis* 1-alkoxy,5-(2′alkoxyethyl)cyclopent-2-ene derivatives, coded "cy" (Fig. 6.5), on the ability of the Varroa olfactory organ to detect stimuli consisting of nurse honeybee volatiles. Utilizing nurse bees' headspace as a positive stimulus, two types of activities of some of these compounds

were identified on the electrophysiological level: (1) decreased responses to honeybee headspace volatiles when the compound was given simultaneously (short-term inhibition) and (2) decreased responses to honeybee headspace volatiles puffed after a mixed compound/headspace stimulus (long-term inhibition).

To reveal structure activity relationship we focused on five racemic substituted cyclopentenes that differ in the length of the ether functional group: **cy**{*1,1*}, **cy**{*2,2*} **cy**{*2,1*}, **cy**{*3,1*}, **cy**{*4,1*} and **cy**{*5,1*}. Only **cy**{*1,1*} proved ineffective; the others showed various degrees of inhibitory effects short and/or long term. One of the most effective compounds was **cy**{*4,1*}. This compound caused significant and dose-dependent inhibition of foreleg responses to honeybee volatiles. It acted on the Varroa olfactory system, by initially stimulating an electrophysiological response, but by later inhibiting subsequent responses to honeybee volatiles (Eliash et al. 2014; Plettner et al. 2017) (Fig. 6.5 and Table 6.2). The mechanism of this inhibition is unclear at this point.

Table 6.2 The effects of the tested compounds on the Varroa nurse sensing, host selection and ability to reach host

Compound name	Compound code	ST inhibition[a]	LT inhibition[b]	Effect on host preference	Effect in host reaching
Hexane	0	–	–	–	nt
1-ethoxy-4-propoxybenzene	**3c**{*2,3*}	–	–	No change	No change
1-methoxy-4-propoxybenzene	**3c**{*1,3*}	–	–	nt[d]	nt
1,4-dimethoxybenzene	**3c**{*1,1*}	ns[c]	+	nt	nt
1-ethoxy,5-(2′ethoxyethyl) cyclopent-2-ene	**cy**{*2,2*}	+	+	Shift	No change
Mixture of cy compounds[e]	HCO- 2169	ns	+	Shift	No change
Cyclopentene-ether- dimethyl	**cy**{*1,1*}	–	–	nt	nt
Cyclopentene-ether-ethyl methyl	**cy**{*2,1*}	+	+	nt	nt
Cyclopentene-ether-propyl methyl	**cy**{*3,1*}	ns	+	nt	nt
Cyclopentene-ether-butyl methyl	**cy**{*4,1*}	+	+	Shift	No change
Cyclopentene-ether-pentyl methyl	**cy**{*5,1*}	ns	+	nt	nt
1-ethoxy-3-ethoxybenzene	**3b**{*2,2*}	ns	+	Shift	No change
1,4-diethoxybenzene	**3c**{*2,2*}	+	ns	Lost	No change
1-ethoxy-2-ethoxybenzene	**3a**{*2,2*}	ns	+	nt	nt
N-Diethyl-meta-toluamide	DEET	ns	+	No change	Decrease

[a]*ST* short-term inhibition-decreased responses to honey bee headspace volatiles when the compound was given simultaneously; long-term inhibition – decreased responses to the following stimulation by honey bee volatiles

[b]*LT* long-term inhibition – decreased responses to the following stimulation by honey bee volatiles

[c]*ns* decrease in response albeit not significant;– no effect; +– statistically significant decrease in response to honeybee headspace ($p < 0.05$)

[d]*nt* not tested

[e]Mixture of **cy**{*1,1*}, **cy**{*2,1*}, **cy**{*3,1*}, **cy**{*4,1*} and **cy**{*5,1*}

So far chemosensory disruption approach has been tested only under controlled laboratory conditions. Future work in the field will reveal to which extent this approach can help control Varroa infestations.

4 Concluding Remarks

It is clear today that sustainable Varroa management must be one that integrates a number of different approaches. Chemosensory methods are specific and well-suited to be implemented alongside other methods to achieve synergistic control. For example, compounds that switch host preference are expected to extend the phoretic phase, thus improving efficacy of other strategies such of soft organic acaricides that can be active only on exposed phoretic mites. In addition, while outside the cells, the mites are more exposed to grooming behavior practiced by the worker bees. The ability of chemosensory disrupting compounds to break the synchronization between the Varroa and the bee, thus promoting the ability of the bees to survive the infestation still remains to be evaluated. In any case, this approach is promising as part of an integrated management scheme of this major apicultural pest. Moreover, emerging knowledge on the structure and function of Varroa chemosensory/olfactory system will allow further development of Varroa specific control agents, such as RNAi based, CRISPR and/or others.

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Chapter 7
Circadian and Seasonal Timing of Insect Olfactory Systems

Sakiko Shiga

Abstract Insects are exposed to cyclic environmental changes caused by regular geophysical events. To cope with physical and biological changes, insects set their activities at an appropriate time of the day and of the year, causing daily rhythm and seasonal rhythm. Using photoperiod and temperature insects adjust their development and reproduction to favorable seasons, and overcome unfavorable ones to enter diapause. During active seasons insect behavior or activity is timed in a fixed period of a day by the circadian clock. Both photoperiodism and daily rhythms employ circadian clock mechanisms. For efficient insect control, consideration of biological timing system is important to determine timing for application of controlling agents or appropriate treatments.

1 Seasonal Rhythm and Daily Rhythm in Insects

Insects are exposed to cyclic environmental changes caused by regular geophysical events. The Earth rotation causes daily changes and its revolution does yearly changes in physical elements, such as light, temperature and humidity. These changes cause biological fluctuations, such as availability of foods or a mate. To cope with physical and biological changes, insects set their activities at an appropriate time of the day and of the year, causing daily rhythm and seasonal rhythm (Beck 1980).

Using photoperiod and temperature insects adjust their development and reproduction to favorable seasons, and overcome unfavorable ones to enter diapause. Diapause has evolved in insects to ensure their survival during unfavorable seasons and synchronize their growth in the population. Diapause is a programmed developmental arrest coupled with endocrine changes and appears at various developmental stages, including the egg, larva, pupa, or adult stage depending on species (Denlinger et al. 2012). Entering diapause or termination of diapause is mediated either in

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response to key stimuli from the environment (facultative diapause) or as a fixed component of ontogeny (obligatory diapause) (Denlinger 2002). Facultative diapause is triggered by changes in environmental conditions, such as photoperiod and temperature, perceived during a developmentally specific sensitive stage that occurs earlier than the stage at which diapause is manifested (Denlinger et al. 2012).

Photoperiod is a main cue to tell coming seasons, and many insects respond to photoperiod to determine alternative developmental programs: non-diapause program and diapause program. For instance larvae of the papilionid butterfy *Papilio xuthus* and tobacco horn worm *Manduca sexta* developing under long days pupate without interruption to the adult, but larvae developing under short days enter diapause at the pupal stage (Fig. 7.1; Rabb 1966; Ichinose 1974; Bell et al. 1975). Diapause pupae overwinter till the next favorable season. Although many lepidotpteran species enter diapause at the pupal stage, species with egg diapause and larval diapause are also known (egg diapause in *Bombyx mori*; larval diapause in *Ostrinia furnacalis*) (Fukuda 1940; Xia et al. 2012). Molecular and endocrine mechanisms controlling diapause with regard to ecdysis or gonadal development have been investigated in many insect species. However, neuronal mechanisms controlling behavioral or sensory change between diapause and nondiapause insects are not well understood, although it is natural to think that physiological states in diapause insects are quite different from nondiapause ones.

Insect behavior or activity is timed in a fixed period of a day by the circadian clock. Calling behavior or mating in moths usually occurs early or late part of the night dependent on species, and this may contribute allochronic differentiation of sexual activities in speciation (Groot 2014). Suppression of activities during an

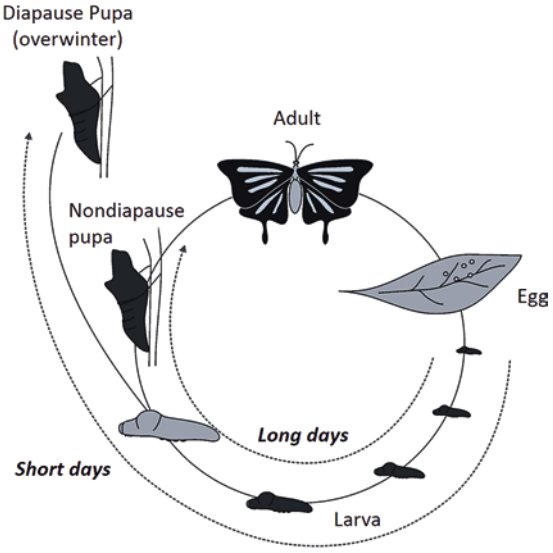

Fig. 7.1 Life cycle of a lepidopteran species which has facultative diapause during the pupal stage. When larvae grow under long-day photoperiod pupae develop without interruption to eclose to adults. When larvae develop under short-day conditions adult development is arrested at the pupal stage. Pupal diapause is facultatively controlled by photoperiod for seasonal adaptation

appropriate time of a day is also adaptive for escape from predation and other environmental dangers (DeCoursey et al. 1997, 2000). An endogenous rhythm with a cycle of a day likely allows organisms to appropriately prepare their physiological states for daily events. Recently a unique periodicity is reported in the large black chafer *Holotrichia parallela* (Coleoptera: Scarabaeidae), an agriculture pest in East Asia. Their activity occurs every 2 days. This rhythm is called circa'bi'dian rhythm, and plausibly driven by the circadian clock (Kawasaki et al. 2017). Males are attracted by the pheromone at sunset time, and mating is observed soon later. In the field, appearance of the chafer on the ground and pheromone production by females occur every 2 days (Yoshioka and Yamasaki 1984; Leal et al. 1993; Kawasaki et al. 2017). Biological significance of the circabidian rhythm should be solved in future study.

2 Circadian Rhythms of Olfactory Behavior

2.1 Circadian-Clock Controlled Olfactory Rhythm

Different kinds of olfactory-based behavior show daily rhythm. Each species has a fixed time of a day for mating, egg laying or foraging (Gruwez et al. 1971; Silvegren et al. 2005; Menon et al. 2014). Daily active phases of behavior are intrinsically set by the circadian clock. Circadian clock (oscillator mechanisms with a periodicity close to 24 h) emanates circadian rhythm (about 24-h behavioral or physiological rhythm; Fig. 7.2). Circadian rhythm freeruns with a period close to 24 h under constant conditions without time cues (endogeneity). There is a time-compensated

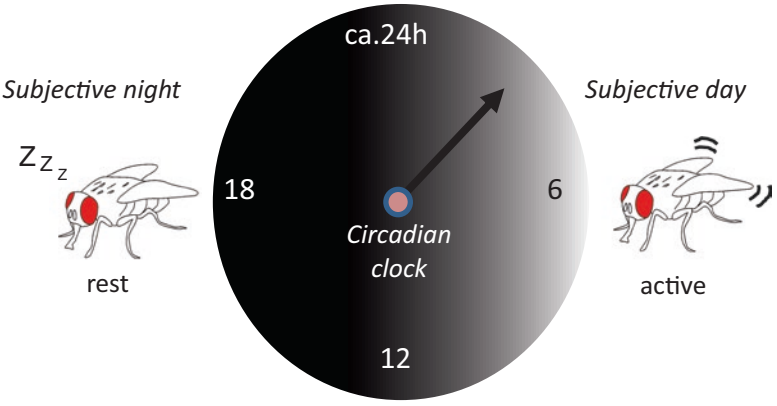

Fig. 7.2 Circadian clock (oscillator mechanisms with a periodicity close to 24 h) controls daily activity rhythm. Diurnal insects such as flies are active during subjective day; nocturnal insects such as cockroaches and crickets are active during subjective night

mechanism to keep the freerunning period close to 24 h under different temperature (temperature compensation). The circadian rhythm entrains to appropriate environmental variables changing with a period of 24 h, thereby attaining a functional phase relationship between internal physiology, and the world outside (entrainability) and the environmental variable is called in German "*zeitgeber*" (Saunders 2002). Endogeneity and entrainability of pheromone source contact behavior and electroantennogram (EAG; the average output of an insect antenna to its brain for a given odorant, see Chaps. 1, 2, and 3, volume 2) rhythm have been shown in the Madeira cockroach *Leucophaea maderae* and the turnip moth, *Agrotis segetum* to suggest that this olfactory rhythm is under control of the circadian clock, although temperature compensation has rarely been shown (Page and Koelling 2003; Rosén et al. 2003).

Many insect species show also daily rhythms in their sexual activities, some species being sexually active early at night, while others are active late at night. This allochronic sexual separation might have arisen to avoid communication interference between closely related species which have overlapping sex pheromone blends (Hardeland 1972; Sakai and Ishida 2001; Schofl et al. 2011; Groot 2014). Species-specific daily rhythm has been shown in calling behavior and sex pheromone production in females, and male responses to the sex pheromone. To our knowledge, the pheromone study in the armyworm *Pseudaletia unipuncta* from Turgeon and McNeil (1982) is the first to demonstrate a circadian rhythm in moth calling behavior.

2.2 Mating and Olfactory Rhythm

In the noctuid moth, Egyptian cotton leafworm, *Spodoptera littoralis* mating and locomotor activities of males show diel rhythm with high activities in the late night to early light phase under 17 h light and 7 h darkness cycles (LD 17:7 h), and the fluctuation persists for at least 1 day in constant darkness (DD) (Silvegren et al. 2005). Mating is generally mediated by sex pheromone released by females in moths. In *S. littoralis*, behavioral response by males to the sex pheromone (pheromone source contact) is also high from late night to early day, and is correlated in time with mating and locomotor activities (Silvegren et al. 2005). It is then thought that male physiological or neuronal activities involved in olfactory behavior is upregulated in late night to early day, and subsequently high responses to the sex pheromone might occur (see Chaps. 1, 2 and 3, volume 2). However, EAG shows that male antennal response to the pheromone decreases late at night and increases after light-on under light and dark (LD) cycles. Under DD conditions EAG rhythm continues with low amplitudes from late subjective night (night phase of the circadian clock) to early subjective day (day phase of the circadian clock) (Merlin et al. 2007). EAG rhythm has an inversive relationship to behavioral rhythm, such as mating and sex pheromone source contact (Fig. 7.3a).

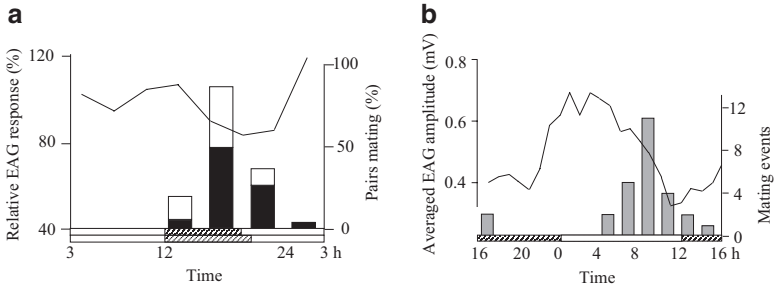

Fig. 7.3 Circadian rhythm of mating and electroantennographic (EAG) response in the moth *Spodoptera littoralis* (**a**) and the cockroach *Leucophaea maderae* (**b**). EAG amplitude and mating activity were examined in constant conditions. EAG amplitude is shown with polygonal lines (left ordinate) and mating frequency is shown with columns (right ordinate). Times on abscissa are expressed in arbitrary values with 12 h set at the light off in the previous light dark (LD) cycles. Hatched and white bars on abscissa show dark and light period in the previous LD cycles. The upper and lower bars in **a** abscissa show conditions for pairs mating and relative EAG response, respectively. White and black column in **a** show successful mating and unsuccessful mating, respectively. Both **a** and **b** show that the olfactory response peak and mating behavioral peak occur at different phase. (Data are from Silvegren et al. (2005), Merlin et al. (2007) and Rymer et al. (2007))

Similar paradoxical results were obtained also in the cockroach *L. maderae* and the fruit fly *Drosophila melanogaster*. In *Leucophaea* cockroaches, males produce and release volatile sex pheromones. Reproductive behavior is initialized by male calling via release of the sex pheromone. The receptive female approaches to mount the male, feeds on the tergal gland secretion, and mating occurs (Roth and Barth 1967; Sreng 1993). In *L. maderae*, the time of copulation onset is rhythmic under LD 12:12 h, with the peak of mating behavior occurring near the light to dark transition. This rhythm persisted in constant dim red illumination and constant temperature. In constant conditions, the freerunning period of the rhythm is slightly less than 24 h, with a peak of copulation at the late subjective day (Rymer et al. 2007) (Fig. 7.3b).

Considering that copulation rhythms could arise from female antennal response rhythm to the sex pheromone components, female EAG rhythm to the sex pheromone has been examined. The EAG amplitudes by response to two sex pheromone components, 3-hydroxy-2-butanone (acetoin) and 3-methyl-2-butenoic acid (senecioic acid), were high in early day and decreased at late day to night period (Rymer et al. 2007) (Fig. 7.3b). Saifullah and Page (2009) simultaneously recorded EAG and single olfactory receptor neuron (ORN) activities of *L. maderae* to show that both EAG amplitude and the spike frequency of ORNs exhibit circadian rhythm with peak amplitude/activities occurring in the subjective day. These two studies showed that the peak of both EAG amplitude and ORN frequency appear in antiphase to the olfactory behavior rhythm. In cockroaches, the EAG amplitude in response to food-related odorant (ethyl acetate, fenchone, and octanol) is also at minimum in the late subjective day to early subjective night (Page and Koelling 2003).

In *D. melanogaster* from a Canton-S (CS) strain, EAG amplitude of the antennae shows robust circadian rhythm (Krishnan et al. 1999). Both to a food odorant, ethyl acetate, and to an odorant that causes behavioral avoidance, benzaldehyde, the EAG amplitude is high in the middle of the night and low during day time under LD 12:12 h. The daily fluctuation continues under constant darkness (DD) with high EAG responses in the middle of subjective night (Krishnan et al. 1999; Tanoue et al. 2004). In contrast, locomotor activity in *D. melanogaster* (CS) is maximal at the time of lights-on and lights-off in LD cycles and the activity peak around the end of subjective day continues in DD (Konopka and Benzer 1971). In addition, in the fly, the EAG rhythm is in antiphase to the olfactory behavioral rhythm: *Drosophila* flies are less sensitive to food odorants or behavioral avoidance molecules during active phase than inactive phase (Krishnan et al. 1999; Tanoue et al. 2004).

One question remains unsolved in moths, cockroaches and flies: how the peripheral olfactory rhythm contributes to olfactory behavior rhythm? It is awaited to perform long term recording from higher ordered neurons in the antennal lobe or mushroom body to examine whether rhythmic patterns of activities are detected in a circadian manner, and how the receptor rhythm is processed into the behavioral rhythm (see Chaps. 1, 2, and 3, volume 2) It is interesting to note that circadian control mechanisms are different between the EAG rhythm and ORN activities as found for instance in *L. maderae*. The EAG amplitude rhythm is controlled by the circadian clock in the optic lobe, whereas ORN frequency rhythm is independent from the optic lobe (Saifullah and Page 2009). The circadian clock driving the locomotor activity rhythm has been found in the optic lobe of *L. maderae* (Nishiitsutsuji-Uwo and Pittendrigh 1968; Page 1982).

The clock in the optic lobe is found in different groups of insects including crickets and flies to reach basic knowledge of the optic lobe as circadian clock loci regulating behavioral rhythm (Tomioka and Chiba 1982, 1984; Helfrich-Förster 2003; Shiga and Numata 2009; Somers et al. 2018). Other circadian clock cells are found in a variety of peripheral tissues (Plautz et al. 1997). Therefore, timing signals come not only from a central clock in the optic lobe but also from a peripheral clock in the insect antennae, probably in the olfactory receptor neuron itself (see Chaps. 1, 2, and 3, volume 2). These timing signals are merged at the antenna for olfactory signals to ascend to the brain at an appropriate time of a day. Then, the optic lobe clock must also be involved to submit timing signal to different brain area in order to drive the locomotor activity that controls specific olfactory behavior.

2.3 Molecular Mechanisms in Drosophila EAG Rhythms

Peripheral clock mechanisms driving EAG rhythm are well described in *D. melanogaster*. The circadian clock machinery maintains circadian time through interlocked feedback loops in expression of a variety of circadian clock genes (Tomioka and

Matsumoto 2015; Somers et al. 2018). In *D. melanogaster*, presence of the circadian clock machinery has been shown in the brain and different peripheral tissues including the antennae using reporter gene driven by promotor of circadian clock gene, namely *period* (Plautz et al. 1997). The antennal clock itself oscillates independently from the brain clock cells and is directly light entrainable. The EAG rhythm disappeared when targeted ablation of clock genes was made in ORNs in a basiconic sensillae which mediate robust electrophysiological responses to ethyl acetate (see Chaps. 1, 2 and 3, volume 2; Tanoue et al. 2004). This indicates that ORNs express both a set of circadian clock proteins and olfactory receptors, and peripheral oscillator cells in the antenna, the ORNs themselves, are required to mediate rhythmic olfactory responses (EAG rhythm).

It was also revealed that antennal ORNs but not central clock cells in the brain are necessary for the EAG rhythms (see Chaps. 1, 2 and 3, volume 2). Targeted rescue of wild-type *cycle*, a circadian clock gene, in the antennal ORN in *cyc01* flies shows these neurons are also sufficient for the olfactory EAG rhythm (Tanoue et al. 2004). How circadian clock genes in the ORNs regulate EAG rhythm? Tanoue et al. (2008) have shown that G protein-coupled receptor kinase 2 (Gprk2) mRNA and protein cycle in a circadian manner with a peak synchronized with a peak of the EAG amplitude. GPRK2 abundance rhythm controls rhythmic accumulation of olfactory receptors in ORN dendrites, which in turn control EAG rhythm (see Chaps. 1, 2, 3, and 4, volume 2).

3 Seasonal Changes in the Insect Olfactory System

3.1 Seasonal Migration and Diapause

Olfactory sensitivity and/or olfactory-based behavior possibly change depending on the season. During the adult stage, mainly two physiological states, migration and diapause, are known to suppress reproduction and sexual activities (Johnson 1963; Saunders 2009). In the same genetic population, these traits are controlled as phenotypic plasticity by response to environmental factors, such as photoperiod, temperature and vegetation (West-Eberhard 2003). Migration is adaptive when the habitat deterioration, in which growth, development or reproduction is not permitted, is merely local so that vegetative activities can be resumed earlier elsewhere than the current habitats. When the deterioration is synchronized throughout the insect habitat range as seasonal temperature depression or dryness, diapause is more appropriate (Kennedy 1961). Compared with diapause states retardation of reproductive onset is small in migrate ones.

3.2 Migration and Reproductive Status

Occurrence of olfactory behavior associated with mating or reproduction is different between non-migrant (reproductive) insects and migrant (vegetative) ones (Kennedy 1961). It has been reported in many lepidopteran species that the migratory behavior is initiated by sexually immature adults (Gatehouse and Zhang 1995). In migrant species, a pre-reproductive period expresses their migratory potential. In moths, the pre-reproductive period is measured as the pre-calling period because only reproductively mature females are observed to'call', i.e. release pheromone (McNeil 1986). Male maturity status can be assessed by observing male's response (brush extension and swiping movements of the abdomen) to the calling females. Sexually matured males show mating behavior towards the female. By these assessments negative correlation between reproductive maturity and flight activity (migratory potential) has been shown (Colvin and Gatehouse 1993). However, the relationship between reproductive status and migratory activity is not very clear. There are species which do not always follow the negative correlation between migration and reproduction. In field collected oriental armyworm *Mythimna separata*, females at the end of summer have little ovarian development whereas a significant proportion of females migrating northward in early summer have developed ovaries and at least one spermatophore, which means they have mated (Zhao et al. 2009).

3.3 Seasonal Migration in Moths

Although seasonal migration is known in many lepidopteran species, it is understood in a very few species how seasonal timing of migration or accompanying reproductive suppression is determined in the life cycle (Chapman et al. 2013). *Danaus plexippus* are famous for seasonal migrants for long distances in North America (Williams et al. 1942; Urquhart and Urquhart 1978). In fall migration they travel for a magnificent distance up to 4000 km from the north-east part of the USA to reach overwintering place in the Central Mexico. Only fall migrants show directional flight and suppress reproductive activities (Williams et al. 1942). In contrast, reproductive summer butterflies do not exhibit directional flight (Zhu et al. 2009). After the fall flight for long distance reproductive arrest continues to be in diapause in Mexico until spring. Using field collected butterflies in Minnesota and Wisconsin (~45°N, ~90°W) from mid-summer to early fall, environmental cues for induction of reproductive diapause was examined in different photoperiod and temperature (Goehring and Oberhauser 2002). Decreasing day lengths and fluctuating temperatures were more effective to induce reproductive diapause than constant day length or temperature. Therefore, combination of photoperiod and temperature is a crucial seasonal cue to induce reproductive suppression, during which olfactory behavior is

potentially stopped. However, environmental factors controlling onset of the long directional flight has not been revealed (Perez and Taylor 2004).

Similarly, the oriental armyworm *M. separata* is a seasonal migrant in Asia. This species reaches high latitudes, where *M. separata* is unable to over-winter, by southerly wind during spring to early summer, and makes a return migration Southward in the fall (Li et al. 1964). Both migrations occur during a short-term arrest of reproductive maturation, and attainment of reproductive maturity suppresses prolonged flight (Han and Gatehouse 1991). Northern incursion in spring to early summer occurs soon after adult emergence and is carried out downwind. In contrast, timing of a return flight by northerly winds before winter is critical for success of their survival, and predicted by decreasing photoperiod and temperature (Han and Gatehouse 1991). In congeneric species *M. unipuncta* of North America, short-day photoperiod delayed a starting day of calling at a given temperature (Delisle and McNeil 1986, 1987).

3.4 Juvenile Hormones and Seasonality

Reproductive diapause is caused by a deficiency of juvenile hormone (JH), and reproduction can be induced by application of JH analogue (JHA) to fall migrants of *D. plexippus* as known in other insect species (Zhu et al. 2009; Denlinger et al. 2012). However, JHA application does not alter directional fall flight, and JHA-applied butterflies still show a directional flight with matured ovaries. Although reproductive diapause and fall migration occur in the same season in *D. plexippus*, underlying endocrine mechanisms inducing reproductive diapause and the migration appear to be different (Zhu et al. 2009). Although olfactory behavior was not examined in fall butterflies with JHA applied, acquirement of reproductive maturity by JHA suggests that JH might also induce olfactory-related mating behaviors, as found for sex pheromone production and release as well as male pheromone responses in moths (Cusson and McNeil 1989; Gadenne 1993; Gadenne et al. 1993; Picimbon et al. 1995).

JH control of olfactory sensitivity has been particularly well examined in the black cutworm *Agrotis ipsilon* (Anton et al. 2007). JH injection causes high sensitivity in pheromone-responding neurons in young males of *A. ipsilon*. In contrast, the sensitivity of neurons responding to plant odors is found to be independent of the JH levels (Greiner et al. 2002). Therefore, JH effects seem to be specific for central processing of sex pheromone, particularly in migrant species of moth such as *A. ipsilon*. In North America, a colonizing generation of *A. ipsilon* migrates northward in the spring and a later generation migrates southward in the autumn (Showers 1997). It has been suggested that *A. ipsilon* enters reproductive diapause before its annual southward migration. However, changes in photoperiod alone do not explain the delayed maturity observed in the field. Other factors alone or in combination with photoperiod may be necessary to induce the reproductive diapause (McNeil et al. 1997; Gemeno and Haynes 2001).

3.5 Seasonal Change of Olfactory Behavior in Riptortus pedestris

Seasonal changes of olfactory sensitivity and/or behavior in correlation with reproductive diapause are also known in non-migrant insect species. In the bean bug *Riptortus pedestris* (Fig. 7.4), pheromone production and attractiveness change between diapause and non-diapause adults (Mizutani et al. 2008; Rahman and Lim 2016). There are five chemical components that have been identified in the aggregation pheromone of *R. pedestris*: a main component (tetradecyl isobutyrate) and four other synergistic chemicals ((*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate, octadecyl isobutyrate and (*E*)-2-hexenyl hexanoate) (Leal et al. 1995; Yasuda et al. 2007a, b). Interestingly, in this odor chemical blend, (*E*)-2-hexenyl (*E*)-2-hexenoate and (*E*)-2-hexenyl (*Z*)-3-hexenoate are detected only in nondiapause males but not in diapause males, suggesting that these components act in sexual communication (Mizutani et al. 2008). In contrast, diapause females of *R. pedestris* are known to be more attracted by the aggregation pheromone, a blend of (E)-2-hexenyl (Z)-3-hexenoate, (E)-2-hexenyl (E)-2-hexenoate, myristyl isobutyrate, and octadecyl isobutyrate at a volumetric ratio of 5.5, 27.6, 38.7, and 14.3%, than nondiapause females, presumably for foraging behavior, i.e. searching for food resource (Rahman and Lim 2016). This is in agreement with pheromone trap data in field studies. The increases in catches of *R. pedestris* attracted to the synthetic pheromone traps always occurred in fall (Endo et al. 2011). These studies indicate that the aggregation pheromones in a Hemipteran alydid species such as *R. pedestris* have two roles: (1) attraction of conspecifics (nondiapause insects) and (2) foraging for suitable host plants (diapause insects). Although further study is necessary to characterize seasonal change of the olfactory response, it is interested to examine how this particular pest insect species, *R. pedestris*, use a mixture of chemicals as aggregation pheromone for seasonally dependent purpose.

Fig. 7.4 The bean bug *Riptortus pedestris*, study model for seasonal variations of pheromone

3.6 Photoperiodism: A Seasonal Timing Mechanism

It is important to consider that photoperiod is crucial to set seasonal timing of activity or diapause in insects. Considering the fact that reproductive behavior is suppressed during diapause at the adult stage, accompanying olfactory behavior could be also controlled by photoperiod. For photoperiodic response photoperiod is received by photoreceptors. Day or night length of a day is measured to distinguish long days and short days (time-measurement system). In many insects, just one short day or long day information is not enough to change endocrine outputs. A certain number of days are required (day-counting system) to affect the insect endocrinological system. Both the time measurement and day counting are called photoperiodic clock system and, circadian clocks are involved in it (Goto 2013; Dolezel 2014). Although photoreceptor organs and endocrine systems have been studied in many species, the molecular and neural mechanism underlying the photoperiodic clock system has not been understood yet. Only necessity of circadian clock genes and clock cells for photoperiodic response have been shown, as described for instance in flies, crickets, and bugs (Pavelka et al. 2003; Sakamoto et al. 2009; Shiga and Numata 2009; Ikeno et al. 2010). Seasonal timing mechanisms controlling olfactory behavior may be under control of JH or other endocrine factors, of which production or secretion is controlled by environmental factors such as diet, photoperiod and temperature as largely described in migrant moth species such as *A. ipsilon* and *P. unipuncta* (Cusson and McNeil 1989; Picimbon et al. 1995). Alternatively, photoperiodic clock mechanisms may directly control the peripheral and central neural components of the insect sensory system (see Chaps. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12, volume 2), including perhaps expression of olfactory receptors (see Chap. 4, volume 2), without mediating the endocrine system (see Chap. 8).

4 Perspectives

The circadian clock mechanisms are extensively studied in insects. How the clock determines the passage of day and night phase to precisely regulate behavioral activities starts to be understood. Molecular oscillatory mechanisms and neural networks are now revealed in many different species (Sandrelli et al. 2008; Tomioka and Matsumoto 2015). However, it remains unsolved how each behavioral rhythm is driven by the clock neuron network. Circadian clock cells are distributed not only in the brain but also throughout the whole body. Coordination mechanisms of the central clock in the brain and peripheral clocks in the receptors or output organs are essential to understand the neuronal mechanisms that drive the rhythm of specific circadian behaviors such as courtship, mating and pheromone release (Somers et al. 2018). Antiphase occurrence of olfactory sensitivity and olfactory behavioral activity is a particular issue to be solved to address desynchronization of

pheromone production/reception pathways for inhibiting chemical communication in insect pests.

The circadian clock system of insects like in other organisms plays also a crucial function in photoperiodic response for seasonal rhythm. It remains unknown how the circadian clock cells, possibly with some other specific neurons, monitor the annual change in day length and use this information for the precise timing of adapted seasonal behavioral response (internal photoperiodic clock system). Only developmental and morphogenetic events such as ecdysis and gonadal maturation have been considered as output of photoperiodic response, and these events have been demonstrated to be caused by humoral factors (Brown et al. 2009). However, these photoperiodic clock centers, which remain to be found, send some specific output signals to the brain center, programming thereby specific variations, seasonal behaviors and/or pheromone activities, should not be excluded. The genetic, molecular and neural mechanisms underlying photoperiodic timing in the control of insect behaviors have not been well characterized. Despite an increasing amount of emerging model organisms including bugs, butterflies, cockroaches, crickets, flies and moths, which exhibit seasonal rhythms, the neural centers and circuits that setting the seasonal time in insects are largely unknown. How the pheromone activities and odor-based behaviors such as aggregation, migration, calling, mating, mate recognition, location of food sources, host-plant selection for suitable oviposition site and/or any other sensory acuity are seasonally controlled is a challenging issue both in chronobiology and insect physiology, crucial to set up sustainable non-insecticide methods of insect pest control.

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Chapter 8
Molecular Basis of Pheromonogenesis Regulation in Moths

J. Joe Hull and Adrien Fónagy

Abstract Sexual communication among the vast majority of moths typically involves the synthesis and release of species-specific, multicomponent blends of sex pheromones (types of insect semiochemicals) by females. These compounds are then interpreted by conspecific males as olfactory cues regarding female reproductive readiness and assist in pinpointing the spatial location of emitting females. Studies by multiple groups using different model systems have shown that most sex pheromones are synthesized *de novo* from acetyl-CoA by functionally specialized cells that comprise the pheromone gland. Although significant progress was made in identifying pheromone components and elucidating their biosynthetic pathways, it wasn't until the advent of modern molecular approaches and the increased availability of genetic resources that a more complete understanding of the molecular basis underlying pheromonogenesis was developed. Pheromonogenesis is regulated by a neuropeptide termed Pheromone Biosynthesis Activating Neuropeptide (PBAN) that acts on a G protein-coupled receptor expressed at the surface of pheromone gland cells. Activation of the PBAN receptor (PBANR) triggers a signal transduction cascade that utilizes an influx of extracellular Ca^{2+} to drive the concerted action of multiple enzymatic steps (i.e. chain-shortening, desaturation, and fatty acyl reduction) that generate the multicomponent pheromone blends specific to each species.

In this chapter, we provide a brief overview of moth sex pheromones before expanding on the molecular mechanisms regulating pheromonogenesis, and conclude by highlighting recent developments in the literature that disrupt/exploit this critical pathway.

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1 Introduction

Sexual communication in most moths is dependent on the female's ability to relay information regarding conspecificity, reproductive status, and location to receptive males. Research in earnest into the underpinnings of this chemical-based sexual communication originated with the seminal structure elucidation study published more than 50 years ago by Butenandt and co-workers (Butenandt et al. 1959). In that study, the authors reported the first chemical identification of an insect sex pheromone, (*E,Z*)-10,12-hexadecadien-1-ol (i.e. bombykol), extracted from 500,000 female silkworm moth (*Bombyx mori*) abdominal glands. Similar herculean efforts lead to the structural identification of sex pheromones from the cabbage looper *Trichoplusia ni* (Berger 1966) and the gypsy moth *Lymantria dispar* (Bierl et al. 1970). Since then, advances in analytical methodologies have facilitated elucidation of sex pheromones from several hundred lepidopteran species (El-Sayed 2014).

Sex pheromones are frequently de novo synthesized as multicomponent blends from acetyl-CoA (a process termed pheromonogenesis) in a specialized organ commonly referred to as the pheromone gland (PG) that is comprised of a single layer of modified epidermal cells between the eighth and ninth abdominal segments (Tillman et al. 1999; Jurenka 2003). Most moths produce Type I sex pheromones, which consist of long, straight chain hydrocarbons ($C_{10–18}$) with varying double bonds and functional modifications (alcohol, aldehyde, or acetate ester) of the carbonyl carbon (Tillman et al. 1999; Jurenka 2003, 2004; Ando et al. 2004). In contrast, Type II sex pheromones account for a small percentage (~15%) of the known lepidopteran compounds and are characterized by unmodified carbonyl carbons that consist of longer polyunsaturated hydrocarbons ($C_{17–23}$) and their epoxide derivatives (Ando et al. 2004; also see Chap. 11 volume 2). Early research on sex pheromone biosynthetic pathways clearly established that fatty acid metabolism intermediates (e.g. palmitic acid/hexadecanoic acid) provided the framework for downstream modifications. Using radiolabeled precursors, researchers were further able to elucidate specific biochemical steps to determine that pheromonogenesis, at least of the Type I pheromones, was derived from the dynamic interplay of selective β-oxidation reactions (i.e. chain-shortening), unique desaturases, and diverse reductive modifications (Bjöstad et al. 1987).

Despite years of foundational biochemical/chemical research, continued interest in the sex pheromone field has been fueled by its clear potential in integrated pest management strategies (Witzgall et al. 2008, 2010) and the ability to offer intriguing evolutionary insights (Roelofs et al. 2002; Lassance et al. 2010; Albre et al. 2013). Recent advances in genome/transcriptome sequencing, expansion of available molecular databases, and the advent of gene knockdown/knockout methodologies (e.g. RNA interference, CRISPR and TALENs) have greatly facilitated our understanding of moth pheromonogenesis at the cellular and molecular levels. This review will focus on the molecular mechanisms governing initiation and propagation

of the signal that drives moth pheromonogenesis with a final section highlighting studies that describe approaches to disrupt and/or exploit this critical pathway.

2 Regulation of Pheromonogenesis

2.1 Hormonal and Neuroendocrine Regulation

2.1.1 Hormonal Regulation

Early observations that the production and release of pheromones in some insect species coincided with female reproductive cycles lead to the hypothesis that pheromone production was hormonally regulated (Barth 1965). The two predominant hormones in insects, juvenile hormone (JH) and 20-hydroxyecdysone (20E), are now recognized as critical regulators of pheromone production in cockroaches (Schal et al. 2003), beetles (Seybold and Vanderwel 2003; Haberer et al. 2010), flies (Wicker-Thomas et al. 2009; Bilen et al. 2013), ants (Cuvillier-Hot et al. 2004; Holman 2012), and wasps (Kelstrup et al. 2014). In moths, the role of JH varies. For relatively long-lived moths, in which sex pheromone production is delayed and activities related to migration and reproduction are asynchronous (i.e. noctuid species such as the armyworm *Pseudaletia unipuncta*, the black cutworm moth *Agrotis ipsilon,* and the cotton bollworm *Heliothis armigera*), JH functions in the control of pheromone production (Cusson and McNeil 1989; Picimbon et al. 1995; Fan et al. 1999; Zhou et al. 2000). In *A. ipsilon*, JH stimulates the release of a peptidergic factor (see Sect. 2.1.2) from production sites in the brain to trigger pheromone production in 4-day old sexually mature females (Picimbon et al. 1995). In species with shorter lifespans, such as *H. armigera*, in which females initiate pheromone production at an earlier stage, JH (JH-II) primes the female PG to respond to the peptidergic factor (Fan et al. 1999). Conversely, JH has also been implicated in pheromonostasis, i.e. suppression of pheromone production after mating (Webster and Cardé 1984). Exogenous JH has been shown to suppress pheromone production in some moth species (Rafaeli and Bober 2005; Bober et al. 2010; Zhang et al. 2014b), and the male-derived sex peptide that mediates the post-mating behavioral switch in *Drosophila* has both allatotropic (triggering JH biosynthesis) and pheromonostatic effects in *H. armigera* (Fan et al. 1999, 2000; Hanin et al. 2012).

Non-JH hormonal factors from the bursa copulatrix have also been reported to be required for pheromone production in the redbanded leafroller (*Argyrotaenia velutinana*), the eastern spruce budworm (*Choristoneura fumiferana*) and the oblique banded leaf roller (*C. rosaceana*) (Fabriàs et al. 1992; Delisle et al. 1999). It has been postulated that the relative importance of the bursa copulatrix in the hormonal regulation of pheromone production may be related to the evolution of enzyme desaturation systems in specific pheromone biosynthetic pathways, as found for instance in tortricid moths (Delisle et al. 1999).

2.1.2 Neuroendocrine and Neural Regulation

Pheromonogenic control in yet other moth species has been shown to proceed by a non-hormonal mechanism, as surgical removal of the *corpora allata* (CA; site of JH synthesis) had no discernible effect on the calling behavior of female saturnid moths (Riddiford and Williams 1971) and injection of CA homogenates also failed to stimulate pheromone production in *Helicoverpa* (*Heliothis) zea* (Raina and Klun 1984). Furthermore, circadian oscillations in pheromone production and emission coinciding with specific points of the day:night cycle (Raina and Klun 1984; Hunt and Haynes 1990; Delisle and Royer 1994; Kamimura and Tatsuki 1994; Gemeno and Haynes 2000; Foster 2000; Rosén 2002; Mazor and Dunkelblum 2005; Fónagy et al. 2011; Bloch et al. 2013; see Chap. 7) and the presence of a circulating pheromonogenic factor in the hemolymph of moths during scotophase (Ichikawa 1998; Jacquin et al. 1994; Ramaswamy et al. 1995) suggested a neuroendocrine component to pheromonogenic regulation. Biochemical analyses using adult *H. zea* females revealed that the factor was a peptide hormone, subsequently purified to homogeneity (see Sect. 2.2.1) and designated Pheromone Biosynthesis Activating Neuropeptide (PBAN), that was present in the brains and subesophageal ganglion (SOG) (Raina and Klun 1984; Raina et al. 1989). Accumulating evidence has supported circadian regulated release of PBAN from the *corpora cardiaca* into the hemolymph for direct pheromonotropic activity on PGs. However, reports describing pheromonotropic activity of a PBAN-like immunoreactive factor in the ventral nerve cord (VNC) and terminal abdominal ganglion, along with impaired pheromone production after severing the VNC suggest regulation may involve a neural component as well (Marco et al. 1996; Iglesias et al. 1998; Teal et al. 1999; Rosén 2002).

Neural signals from the VNC and depletion of sperm in the spermatheca are also important post-copulatory factors that regulate post-mating inhibition of pheromone production in polyandrous moths (Delisle et al. 2000; Delisle and Simard 2002). Mated females of polyandrous (multiple matings) species usually display a refractory period to reproduction after mating, which is largely due to the transfer of male humoral factors (sperm and seminal fluid) during copulation. Some of these male factors have short-term effects, whereas others can induce long-term suppression of female receptivity, as described in both butterflies and moths (Wedell 2005).

2.2 Purification and Characterization of the Pheromone Biosynthesis Activating Neuropeptide (PBAN)

2.2.1 HPLC-Based Identification of PBAN

Determination that the moth phermonotropic factor (i.e. PBAN) was a peptide hormone present in the brains and SOG of adult *H. zea* females facilitated HPLC (high-performance liquid chromatography) purification of the 33-amino acid PBAN from

2500 *H. zea* female brain-SOG complexes (Raina et al. 1989). Neuropeptides with similar functionalities and moderate overall sequence homology were likewise purified to homogeneity and sequenced from *B. mori* (Kitamura et al. 1989, 1990) and *L. dispar* (Masler et al. 1994). Consistent with its presumed role as the cue driving circadian oscillations in pheromone production, PBAN levels in both the brain and hemolymph fluctuate in accordance with photoperiod (Rafaeli et al. 1991, 1993; Rafaeli 1994; Ramaswamy et al. 1995; Iglesias et al. 2002; Nagalakshmi et al. 2007; Závodská et al. 2009). All PBANs have a conserved FxPRL-NH_2 (Phe-Xxx-Pro-Arg-Leu-amide) C-terminal pentapeptide motif that is critical for pheromonotropic activity (Raina and Kempe 1990; Kitamura et al. 1989). In addition, these pheromonotropic peptides exhibited species cross-reactivity as well as functional cross-reactivity with locust myotropins and tachykinins (Kuniyoshi et al. 1992; Fónagy et al. 1992a, b; Nachman et al. 1993a, b), suggesting that the cognate FxPRL-NH_2 peptide receptors were also similar.

2.2.2 Structure-Function Analysis of PBAN

Initial structure-function analyses of PBAN examined the pheromonotropic efficacy of peptide fragments generated either as a series of N-terminally truncated synthetic peptides (Raina and Kempe 1990) or endoproteinase Glu-C fragments (Kitamura et al. 1989). In both studies, the minimal sequence needed to stimulate pheromone production consisted of the C-terminal pentapeptide core (i.e. FxPRL). Comparison of amidated, hydroxylated, and methyl ester versions of the pentapeptide revealed the critical importance of the C-terminal amide (Kitamura et al. 1989; Kuniyoshi et al. 1992; Nagasawa et al. 1994). Sequential amino acid substitution of the core pentapeptide motif in *B. mori* (FSPRL-NH_2) revealed that Phe and Ser could be replaced with similar residues with little disruption of pheromonotropic activity, whereas Pro, Arg, and Leu could not (Kuniyoshi et al. 1991). Comparison of the pheromonotropic efficacies of FxPRL-NH_2 peptides from diverse species provided further insights into the structure-function relationships and suggested that the variable "x" position had greater pheromonotropic properties if occupied by Thr compared to Val, Ser, or Gly (Abernathy et al. 1995). More recent structure-function analyses revealed that the positively charged basic Arg (R; two positions from the C terminus) is the most critical residue within the hexapeptide motif (Kim et al. 2008; Kawai et al. 2012). It is followed in importance by the branched chain Leu, aromatic Phe, and then to a lesser extent by the other residues (Kim et al. 2008).

To provide greater insights into the role the individual residues in the C-terminal pentapeptide motif might play in receptor activation, Nachman and co-workers used nuclear magnetic resonance (NMR) spectroscopy, circular dichroism, and molecular dynamics simulations to determine that a cyclic analog of the pentapeptide adopts a C-terminal β turn in solution (Nachman et al. 1991). The analog, which introduced significant conformation constraints and increased the overall rigidity of the pentapeptide, retained biological activity, indicating that this conformation is crucial for receptor activation. Molecular simulations using the linear pentapeptide

active core suggested the conformation was not specific to the cyclization process. Subsequent NMR analyses of a hexapeptide (TFSPRL-NH_2) analog and the full-length *H. zea* PBAN confirmed that the peptide assumes a C-terminal type I′ β turn in solution (Wang et al. 1994; Clark and Prestwich 1996). A more recent NMR study of an 18-amino acid pheromonotropin from *Pseudaletia separata* characterized by a C-terminal FxPRL-NH_2 revealed an extended β sheet structure devoid of the previously identified β turn (Bhattacharya et al. 2015). However, that study was performed in water as opposed to a more polar solvent (e.g. trifluoroethanol/water or dimethyl sulfoxide/water) that would presumably more accurately mimic the lipid bilayer environment in which the cell surface receptors are embedded.

2.3 Molecular-Based identification of PBAN

2.3.1 PBAN Transcripts

Following purification of the respective PBANs, cloning methods employing sequence information provided by the isolated peptides facilitated molecular elucidation of the *B. mori* and *H. zea* PBAN gene products (Davis et al. 1992; Kawano et al. 1992; Sato et al. 1993; Ma et al. 1994). In both instances, post-translational proteolytic processing of the encoded open reading frames was predicted to yield the respective PBANs and four additional peptides with C-terminal FxPRL-NH_2 motifs identified as diapause hormone (DH) and α, β, and γ subesophageal neuropeptides (i.e. SGNPs). Among the four additional peptides, DH had previously been isolated to homogeneity and shown to function in embryonic diapause (Imai et al. 1991). Synthetic α, β, and γ SGNPs were reported to have pheromonotropic activity in *H. zea* (Ma et al. 1994), but in *B. mori* the α and γ SGNPs were less effective than PBAN (β SGNP was comparable) at stimulating pheromone production and all three were less potent than DH in diapause induction (Sato et al. 1993). Later studies using PBANR receptors heterologously expressed in *Xenopus* oocytes, however, reported that the three SGNPs were more potent than PBAN in generating chloride currents (Watanabe et al. 2007).

Organization of the FxPRL-NH_2 open reading frames is conserved in both the *B. mori* and *H. zea* transcripts with the DH sequence downstream of the signal peptide followed by the α and β SGNPs, PBAN, and then γ SGNP. Since initial cloning, PBAN-encoding cDNAs with similar sequence architecture have been published for 22 lepidopterans with additional sequences deposited in GenBank or the Transcriptome Shotgun Assembly (TSA) sequence databases (Table 8.1) with most of the peptides composed of 33 residues (Fig. 8.1). Outliers include the *Ascotis selenaria cretacea* (Japanese giant looper) PBAN, which is 27 amino acids, and the 37 amino acid *Omphisa fuscidentalis* PBAN. A second 37 amino acid PBAN recently identified in *Ostrinia nubilalis* suggests close conservation of PBAN gene architecture between the closely related crambid subfamilies Pyraustinae and Spilomelinae (Fodor et al. 2017). The *A. s. cretacea* PBAN transcript is also unique

Table 8.1 Accession numbers for PBAN and PBANR sequences identified in lepidopteran species

PBAN		PBANR	
Species	GenBank protein accession no.	Species	GenBank protein accession no.
Published sequences			
Adoxophyes sp.	AAK72980	*Agrotis segetum*	AID66638
Agrotis ipsilon	CAA08774/O76818	*Bombyx mori*	AEX31546, AEX15646, AEX15643/BAD44726, AEX15640
Antheraea pernyi	AAR17699	*Helicoverpa armigera*	AEX31547, AEX15647/AAW47417, AEX15644, AEX15641
Ascotis selenaria cretacea	BAF64458	*Helicoverpa zea*	AAP93921, AEO17028, AFP19101
Bombyx mandarina	AAM88285	*Heliothis peltigera*	AEQ33641
Bombyx mori	BAA05954/AAB24327	*Heliothis virescens*	ABU93812, ABU93813, ABV58013
Chlumetia transversa	AIY72749	*Mamestra brassicae*	ARO85771-ARO85773
Clostera anastomosis	ABR04093	*Ostrinia nubilalis*	AGL12066-AGL12068
Helicoverpa armigera	AAM43840/AAL05596/AAQ82626	*Plutella xylostella*	AAY34744/AEP25401
Helicoverpa assulta	AAC64293	*Pseudaletia separata*	AEX31548, AEX15648, AEX15645, AEX15642
Helicoverpa zea	P11159/AAA20661	*Spodoptera exigua*	ABY62317
Heliothis virescens	AAO20095	*Spodoptera littoralis*	ABD52277
Holcocerus hippophaecolus	n/a[a]		
Mamestra brassicae	AAC02094		
Manduca sexta	AAO18192		
Maruca vitrata	AGI96545		
Omphisa fuscidentalis	AFP87384		
Ostrinia nubilalis	AOY34014		
Plutella xylostella	AAX99220		
Samia cynthia ricini	AAP41132		

(continued)

Table 8.1 (continued)

PBAN		PBANR	
Species	GenBank protein accession no.	Species	GenBank protein accession no.
Spodoptera exigua	AAT64424/AAR87744		
Spodoptera littoralis	AAK84160		
Spodoptera litura	AJT60314		
Unpublished sequences (GenBank annotations only)			
Chilo suppressalis	ALM30314	*Chilo suppressalis*	ALM88337-ALM88338
Omphisa fuscidentalis	AFP87384	*Manduca sexta*	ACQ90219-ACQ90222
Orgyia thyellina	BAE94185	*Spodoptera litura*	AJW32184
Ostrinia furnacalis	BAQ21230		
Genome Annotations			
Amyelois transitella	XP_013189838	*Amyelois transitella*	XP_013187133
Danaus plexippus	EHJ67284	*Papilio machaon*	XP_014362489,XP_014362488,XP_014362487
Papilio machaon	XP_014371142	*Papilio polytes*	XP_013142894,XP_013142893,XP_013142892
Papilio polytes	XP_013144402	*Papilio xuthus*	XP_013176026, XP_013176019, XP_013176012
Papilio xuthus	XP_013168299/XP_013163175/ XP_013168300		
Transcriptome Shotgun Assembliesb			
Athetis lepigone	GARB01004345	*Actias selene*	GBZL01006651
Biston suppressaria	GCJP01035652	*Antheraea yamamai*	GBZJ01027120
Chilo suppressalis	GAJS01037377	*Athetis lepigone*	GARB01028884
Dyseriocrania subpurpurella	GASY02017090	*Biston suppressaria*	GCJP01052341
Nemophora degeerella	GATC02010886	*Cadra cautella*	GBXH01027379
Papilio zelicaon	JP623453	*Helicoverpa assulta*	GBTA01046701/GBTA01046700

Polyommatus icarus	GAST02017042	*Nemophora degeerella*	GATC02017805
Spodoptera frugiperda	GESP01042864.1	*Ostrinia furnacalis*	GAQJ01060384
Triodia sylvina	GAVB02014270	*Parides eurimedes*	GAXH02029056
Yponomeuta evonymellus	GASG02034409	*Polyommatus icarus*	GAST02014754
		Spodoptera frugiperda	GESP01096852
		Yponomeuta evonymellus	GASG02024048

[a]See Li J, Zhou J, Sun R, et al (2013) Arch Insect Biochem Physiol 82:183–195. doi: 10.1002/arch.21084
[b]tBLASTn against TSA archive (08/28/2016) using *B. mori* PBAN (AAB24327) with *e* value $<1e^{-05}$ or PBANR (BAD44726) *e* value $<1e^{-60}$

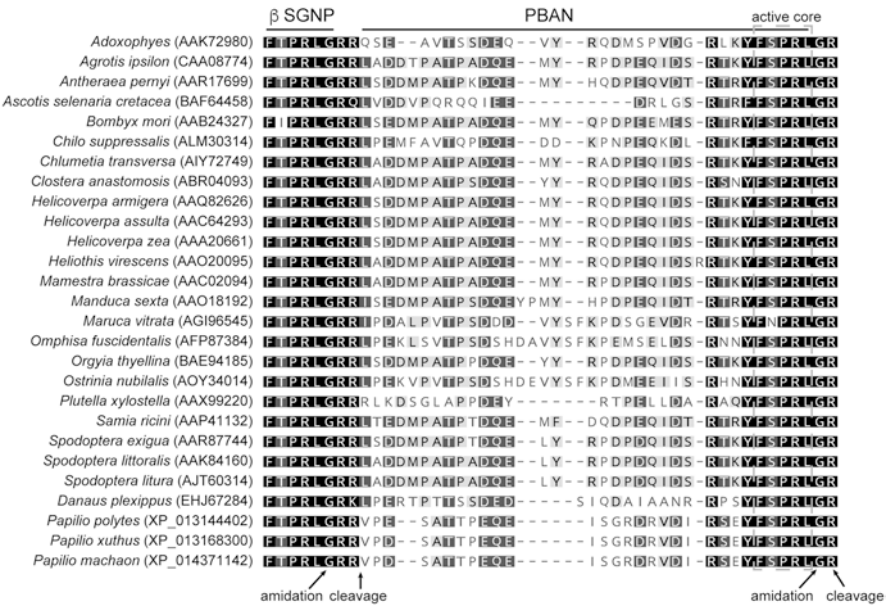

Fig. 8.1 Multiple sequence alignment of PBAN coding sequences from diverse lepidopteran species. The alignment was made using MUSCLE implemented in Geneious 7. Sequences correspond to the portion of the DH/PBAN transcript from the FxPRL portion of the β SGNP through the predicted proteolytic cleavage site at the C terminus of the PBAN sequence. Predicted cleavage and amidation sites are indicated. The essential FxPRL active core of PBAN is indicated by the dashed grey lines. Genome-based butterfly sequences are clustered at the bottom of the alignment. Protein accession numbers are indicated in parentheses

in that it generates a fused β SGNP/PBAN with a double FxPRL motif (Kawai et al. 2007). Alignment of multiple lepidopteran PBAN sequences revealed that the variable position in the FxPRL-NH_2 motif reported to have an effect on pheromonotropic activity (Abernathy et al. 1995) is a conserved Ser (Fig. 8.1). The exception is *Maruca vitrata* (legume pod borer), which has Asn, an uncharged polar residue with a bulkier sidechain than Ser (Chang and Ramasamy 2014). Furthermore, all of the published PBAN cDNAs to date contain a dibasic KK motif upstream of the α SGNP sequence. While KK cleavage has been reported to be infrequent (Veenstra 2000), proteolytic processing of the PBAN prepropeptides was confirmed via HPLC-based fractionation of *B. mori* SOGs (Sato et al. 1993) and MALDI (matrix-assisted laser desorption/ionization) mass spectrometry of individual *H. zea* SOG neuronal clusters (Ma et al. 2000).

The presence of PBAN sequence (and/or prepropeptide) variants was initially described in *B. mori* following HPLC-based purification of two peptides (PBAN-I and PBAN-II) with pheromonotropic activity that differed from one another by a single N-terminal Arg residue (Kitamura et al. 1989, 1990). Since then potential sequence variants have been deposited with the NCBI database for a number of species including *B. mori* (three point mutations between AAB24327 and

BAA05954-K109 N, M139I, and E146V), *Spodoptera litura* (one point mutation between AJT60314 and AKT95050-E53G), and *Helicoverpa armigera* (three point mutations between AAL05596 and AAM43840-deletion of N3, insertion of G before G30, and M179I).

At this point, it is uncertain if these variants represent population differences, are differentially expressed variants, or are merely the result of sequence errors introduced during cloning. However, differentially expressed PBAN prepropeptide transcript variants have been reported in the sand fly *Phlebotomus papatasi* (Choi et al. 2015) and are suggested based on a band doublet observed on an RT-PCR gel of fire ant thoraces (Choi et al. 2011). More recently, transcripts that vary in the length and composition of their 3′UTRs (untranslated regions) have been identified in *O. nubilalis* (Fodor et al. 2017).

2.3.2 PBAN Gene Structure

The lepidopteran PBAN genomic structure is conserved with PBAN genes in *B. mori* (Xu et al. 1995), *H. armigera* (Zhang et al. 2005), *M. vitrata* (Chang and Ramasamy 2014), and *Clostera anastomosis* (Jing et al. 2007) encompassing six exons with identical exon coding (Fig. 8.2). Exon one encodes the signal peptide and a portion of DH, exon two the remaining portion of DH, exon three an uncharacterized peptidergic sequence, exon four the α and β SGNPs and a portion of PBAN, and exon five the remaining portion of PBAN and γ SGNP. The stop codon is located in exon six. Splicing of all four genes follows the GT-AG rule and utilizes 0, 2, 1, 2, 1 phasing; however, despite the similarities, the overall sizes of the genes differ with varying intron lengths (Fig. 8.2). The *O. nubilalis* PBAN was recently reported to have the same genomic structure (Fodor et al. 2017).

Limited promoter analyses, which focused on elucidating how DH expression was regulated in relation to embryonic diapause as opposed to pheromonogenesis, identified potential differences in transcription between the *B. mori* and *H. armigera* genes. POU-M2, a eukaryotic transcription factor with a bipartite DNA binding domain implicated in neuroendocrine function, activated expression from the *B. mori* PBAN promoter *in vitro* but failed to do so with a conserved region of the *H. armigera* promoter (Zhang et al. 2004a, 2005). In contrast, an E-box element (CAGCTG) present in the *H. armigera* promoter was reported to be critical for transcriptional activation (Hong et al. 2006), which was dependent on co-ordinate interactions with upstream activating and inhibitory regions. Taken together, the findings suggest that the two species utilize variations in transcriptional regulation to drive the respective differences in diapause programs. Additionally, an ecdysone response element was identified in the promoter region of the *B. mori* PBAN gene (Xu et al. 1995). While ecdysteroids have not been associated with diapause control, they are critical regulators of lepidopteran reproduction (Van Wielendaele et al. 2013; De Loof et al. 2016). Consequently, the response element may link PBAN transcription with reproductive competence; however, the role it has in pheromonogenesis remains to be revealed.

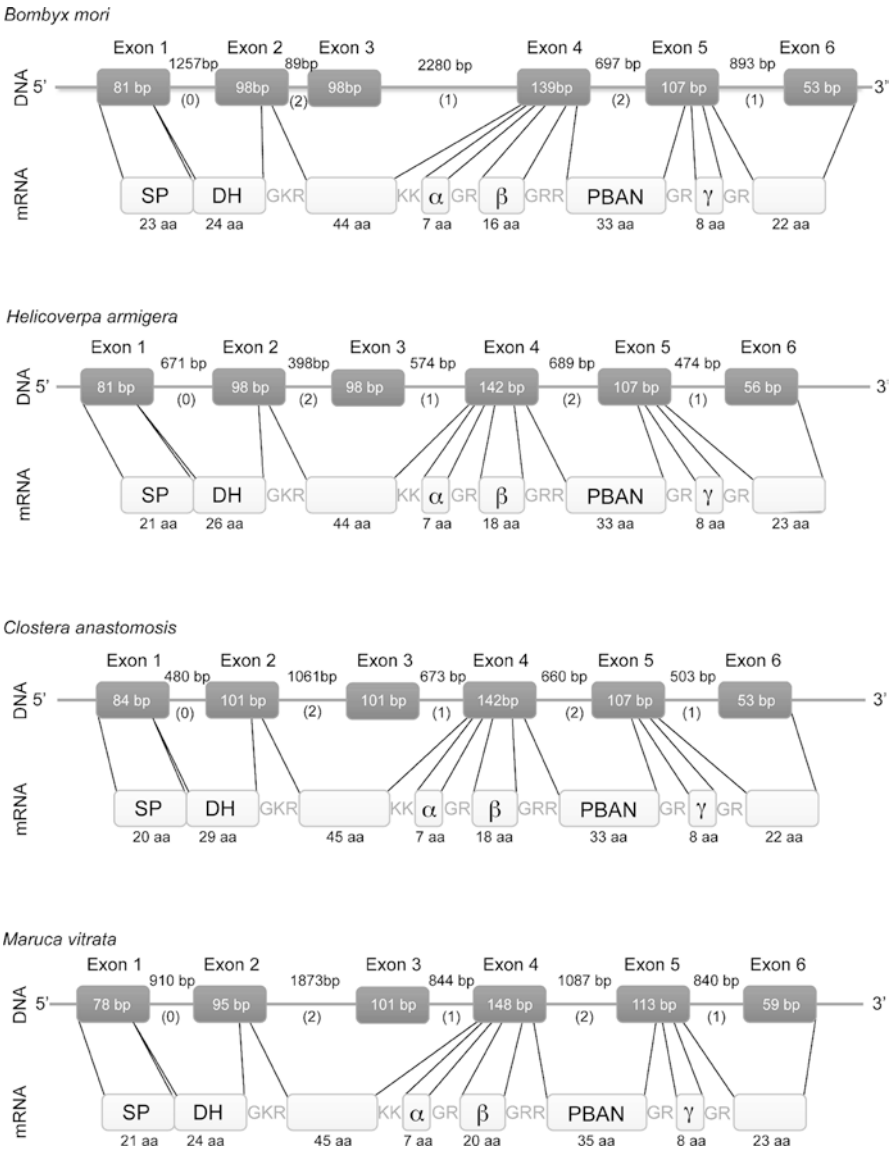

Fig. 8.2 Genomic organization of the DH-PBAN gene in four moth species. Schematic comparison of genomic DNA and the translated peptides for the DH-PBAN gene in *Bombyx mori* (Xu et al. 1995), *Helicoverpa zea* (Zhang et al. 2005), *Clostera anastomosis* (Jing et al. 2007), and *Maruca vitrata* (Chang and Ramasamy 2014). Darker shaded boxes indicate exons, whereas lighter shaded boxes indicate the encoded peptides. Horizontal solid lines represent introns with the corresponding intron phase in parentheses. GKR, KK, GR, and GRR indicate probable endoproteolytic cleavage sites. SP - signal peptide; DH-diapause hormone; α-α SGNP; β-β SGNP; PBAN - pheromone biosynthesis activating neuropeptide; γ-γ SGNP. Note, while the sizes of exons and introns are indicated, the models are not drawn to scale

2.4 *Other FxPRL-NH_2 Peptides*

The critical C-terminal pentapeptide is now recognized as a defining feature of the PBAN/pyrokinin (FxPRL) family of pleiotropic neuropeptides present throughout Insecta and includes pyrokinins, PBANs, myotropins, DH, and the α, β, and γ SGNPs (Predel and Nachman 2006; Jurenka and Nusawardani 2011; Altstein et al. 2013; Jurenka 2015; Yaginuma and Niimi 2015). In addition to the pheromonotropic effects in moths, FxPRL-NH_2 peptides also regulate the induction of cuticular melanization in moth larvae (Matsumoto et al. 1992; Altstein et al. 1996), the induction of embryonic diapause and seasonal polyphenism in moths (Imai et al. 1991; Uehara et al. 2011), the termination of pupal diapause in heliothine moths (Xu and Denlinger 2003; Zhang et al. 2004b, c; Zhao et al. 2004), prothoracic gland ecdysteroidogenesis (Zhang et al. 2004c; Watanabe et al. 2007), visceral muscle contraction in cockroaches (Holman et al. 1986; Nachman et al. 1986; Predel et al. 2001), acceleration of puparium formation in flies (Zdárek and Nachman 1997; Zdárek et al. 1998, 2002, 2004), production of fatty acid components in male *H. armigera* hair-pencil aedeagus complexes (Bober and Rafaeli 2010), and the biosynthesis of trail pheromones in *Solenopsis invicta* (Choi and Vander Meer 2012). This multifunctionality is similar to the structural variation described for the chemosensory protein (CSP) family of multi-function transporters, which are widely expressed in diverse tissues including the PG (see Chaps. 6, 9, and 10, volume 2; Xuan et al. 2014, 2016; Picimbon 2017).

PBAN control of pheromonogenesis, however, is not ubiquitous throughout the moths (Tang et al. 1989; Subchev and Jurenka 2001; Fujii et al. 2010) nor is it specific to moths that produce Type I pheromone components (albeit our knowledge of this system is more complete) as it has been reported to regulate production of Type II pheromones in the giant looper *A. s. cretacea* (Wei et al. 2004; Fujii et al. 2007). Furthermore, some Lepidopteran species such as *T. ni* do not exhibit diel periodicity in pheromone production (Hunt and Haynes 1990; also see references in Rafaeli and Jurenka 2003; Altstein 2004a), and as such would be expected to have little need for PBAN-mediated regulation. However, *T. ni* brain extracts were found to have pheromonotropic activities in other moth species (Tang et al. 1989). Since then it has become apparent that PBAN is a pleiotropic regulator of diverse activities (see above). Indeed, the elucidated primary structure of the HPLC-purified *B. mori* peptide responsible for larval cuticular melanization (i.e. melanization and reddish coloration hormone) was identical to the PBAN sequence (Matsumoto et al. 1990).

2.5 *Identification of the PBAN Receptor (PBANR)*

2.5.1 PBANR: Early Studies

The involvement of a cell surface receptor that mediates the pheromonotropic effects of PBAN was demonstrated early on following direct stimulation of dissected PGs by PBAN (Soroker and Rafaeli 1989; Jurenka et al. 1991b; Fónagy et al. 1992a, c). Pharmacological profiling with NaF (sodium fluoride), a potent G protein activator that had pheromonotropic effects (Rafaeli and Gileadi 1996a, b) further pointed to the involvement of a G protein-coupled receptor (GPCR). The photoaffinity labeling of a ~50 kDa membrane protein in *H. armigera* PG cells with a biotinylated PBAN analog provided incontrovertible evidence of a PG-derived cell surface protein (Rafaeli and Gileadi 1999; Rafaeli et al. 2003, 2007). However, molecular identification of the moth PBAN receptor (PBANR) ultimately depended on publication of the *Drosophila melanogaster* genome (Adams et al. 2000).

2.5.2 Homology-Based Cloning of PBANR

Sequence homologies between mammalian receptors and putative GPCRs in the *Drosophila* genome led researchers to propose that co-evolution of receptors and their ligands would yield closely aligned receptor families (Hewes and Taghert 2001). Based on this hypothesis, similarities in the active core of FxPRL-NH_2 peptides and neuromedin U (FRPRN-NH_2) suggested that the respective receptors are evolutionarily related. Functional analyses demonstrated that three *Drosophila* GPCRs (CG8784, CG8795, and CG9918) that clustered in phylogenetic analyses with the neuromedin U receptor (NmUR) clade were activated to varying degrees by FxPRL-NH_2 peptides (Park et al. 2002). A subsequent study reported pheromonotropic effects of mammalian NmU in *H. zea*, which further bolstered the receptor co-evolution hypothesis and showed that homology-based methods could be used to clone receptors from the NmUR clade (Choi et al. 2003). The *H. zea* GPCR identified in that study was amplified from PG cDNAs and, when heterologously expressed in cultured Sf9 cells, dose-dependently triggered an influx of extracellular Ca^{2+} in response to synthetic *H. zea* PBAN. This was interpreted as evidence that the authors had identified the first PBANR (i.e. HelzePBANR). Using a similar approach, the *B. mori* PBANR (BommoPBANR) was likewise cloned from PG cDNAs. BommoPBANR mobilized extracellular Ca^{2+} in response to PBAN stimulation, had significant sequence similarity with NmUR homologs, and was up-regulated on the day preceding adult eclosion (Hull et al. 2004), a time period that coincides with *B. mori* pheromonogenesis (Matsumoto et al. 2007, 2010).

2.5.3 The Complexity of PBANR

Identification of PBANR Variants

Perplexingly, the ~50 kDa protein labeled with the biotinylated PBAN analog in the intersegmental membranes that comprise the *H. armigera* PG (Rafaeli and Gileadi 1999; Rafaeli et al. 2003, 2007) was closer in size to BommoPBANR (45.9 kDa) than the smaller HelzePBANR (38.6 kDa). Despite presumably mediating similar biological responses and significant sequence identity through the seventh transmembrane domain (TM7), BommoPBANR was differentiated by the presence of a 67-aa C-terminal extension critical for ligand-induced internalization (Hull et al. 2004, 2005), an endocytotic mechanism associated with GPCR feedback regulation and desensitization (Moore et al. 2007; Marchese et al. 2008). Further confounding the issue, PBANRs subsequently identified in *H. armigera* (Rafaeli et al. 2007), *S. littoralis* (Zheng et al. 2007), *Spodoptera exigua* (Cheng et al. 2010), and *Plutella xylostella* (Lee et al. 2011) also lacked the C-terminal extension, suggesting feedback regulation of these receptors differed from BommoPBANR. The prevalence of the "short" PBANRs raised questions concerning the evolutionary significance of the BommoPBANR extension. Initially, comparisons were made with type I gonadotropin-releasing hormone receptors in which non-mammalian receptors have a C-terminal tail and undergo rapid ligand-induced internalization, whereas mammalian receptors lack the extended C terminus and have significantly different internalization kinetics (Pawson et al. 1998; McArdle et al. 2002). The potential biological significance of the "short" and "long" PBANRs also led to speculation that the varied C-terminal lengths reflected differences in the importance of the second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) in the respective species. The identification of three PBANR variants concomitantly expressed in *Helicoverpa virescens* (referred to as HelviPBANR A-C) with a conserved N-terminal sequence, but with differing C-terminal lengths (Kim et al. 2008), further underscored the complexity of the PBAN signaling system. Similar to BommoPBANR, the HelviPBANR-C variant has an extended C terminus and contains a defined internalization motif (see 2.7.7), whereas the C-terminal end of the HelviPBANR-A variant resembles HelzePBANR. Moreover, HelviPBANR-C was preferentially amplified from PGs and generated robust Ca^{2+} mobilization responses following stimulation with *H. zea* PBAN. In contrast, the other two variants were amplified from larval tissues and failed to respond to the concentration of the synthetic PBAN assayed (Kim et al. 2008). These results initiated a re-evaluation of the species-specific "short" and "long" PBANR paradigm.

Using modified cloning methods, multiple PBANR variants (PBANR-As, -A, -B, and -C) were amplified from PGs of *B. mori*, *H. zea*, *H. armigera*, and *P. separata* (also referred to as *Mythimna separata*) that differed only in the length of their respective C-terminal ends (Fig. 8.3a). Similar to *H. virescens*, the most abundant PG transcripts were the "long" PBANR-C variants (Fig. 8.3b), all of which underwent ligand-induced internalization (Lee et al. 2012a). In contrast, the "short" PBANR-A variants were less abundant, mobilized extracellular Ca^{2+} poorly in

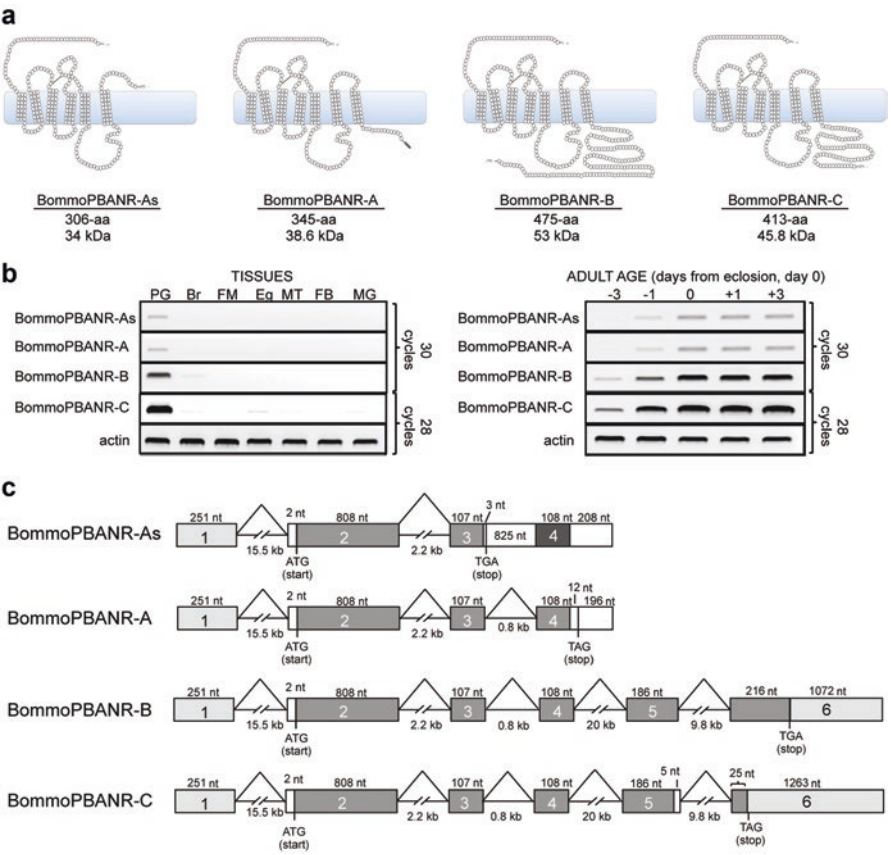

Fig. 8.3 Identification of multiple PBANR variants in *Bombyx mori* pheromone gland. (**a**) Schematic diagram depicting the sizes and structures of the various BommoPBANR variants cloned. (**b**) RT-PCR based expression profile of BommoPBANR variants in various tissues and at varying developmental time points relative to adult emergence (day 0). Abbreviations: *PG* pheromone gland, *Br* brain, *FM* flight muscle, *Eg* unfertilized egg, *MT* Malpighian tubule, *FB* fat body, *MG* midgut. This research was originally published in Frontiers of Endocrinology. Lee et al. (2012a). (**c**) Genomic organization and alternative splicing of the *Bombyx mori* PBANR gene. The four BommoPBANR variants (As, **A**, **B**, and **C**) are depicted. Light grey shading corresponds to untranslated exons, medium grey to translated exons, and dark grey to a non-translated exon that is unique to the As variant. Non-shaded boxes indicate non-spliced intronic sequences. Initiation (ATG) and stop sites (TGA or TAG) are indicated by their respective codons. (Figure adapted from Lee et al. 2012a)

response to a range of PBAN concentrations, and exhibited different internalization kinetics (Lee et al. 2012a, b). Previous preferential amplification of the shorter variants (Choi et al. 2003; Rafaeli et al. 2007; Cheng et al. 2010; Lee et al. 2011) was attributed to the high GC content (55–80%) of the extended C-terminal ends (Lee et al. 2012a), which can reduce PCR amplification efficiencies by serving as pause

or termination sites (McDowell et al. 1998). Thus it is now apparent that the ~50 kDa protein labeled with the biotinylated PBAN analog (Rafaeli and Gileadi 1999; Rafaeli et al. 2003, 2007) was most likely the HelarPBANR-C variant (51.1 kDa) rather than a glycosylated HelarPBANR-A variant (38.7 kDa) as first proposed.

PBANR Variants Arise from Alternative Splicing

Alternative splicing has been extensively documented for GPCRs (Minneman 2001; Markovic and Challiss 2009) and is one of the principal means by which organisms generate functional protein diversity in a temporal- and/or tissue-dependent manner. The modular aspect of the PBANR variants (i.e. variation specific to the C terminus) is consistent with alternative splicing. The availability of the *B. mori* genome (Mita et al. 2004; Duan et al. 2010) allowed further exploration of that hypothesis. BommoPBANR localizes to a >50 kb segment of chromosome 12 and encompasses six exons and five introns (Fig. 8.3c). The N terminus through the last transmembrane domain (i.e. TM7) are encoded on exons 2–4, the C terminus on exons 5–6, and the 5′ untranslated region on exon 1. The introduction of premature stop codons following retention of introns 3 or 4 yields the BommoPBANR-As and BommoPBANR-A variants, respectively. BommoPBANR-C arises from a five-nucleotide frame shift insertion at the 3′ end of exon 5 that changes codons for the remaining ten amino acids (residues 404–413) and introduces a stop codon that generates a 67 amino acid C terminus. In contrast, BommoPBANR-B is generated from conventional splicing of exons 2–6 (Lee et al. 2012a). As more lepidopteran genomes become available, it will be interesting to see if the splicing mechanisms that generate the BommoPBANR variants are conserved in other species and what cellular/transcriptional factors trigger those splicing events.

PBANR Variants: Fine-Tuning the PBAN Signal?

To date, PBANRs have been reported or annotated in 15 species (Table 8.1) with multiple variants present in *O. nubilalis* (Nusawardani et al. 2013), *Manduca sexta* (FJ240221-FJ240224), *Chilo suppressalis* (KT031039-KT031040), *Mamestra brassicae* (Fodor et al. 2018), and based on genomic sequencing data, three *Papilio* species.

While the biological significance of concomitant expression of multiple PBANRs in PGs remains to be determined, one possibility is that they provide a mechanism for fine-tuning cellular responsiveness to the respective PBAN signals. In one model, nominally non-responsive PBANR-A receptors expressed at the cell surface could potentially function as ligand sinks that compete with PBANR-C for ligand binding. The net result would be less bioactive peptide available to trigger the cellular response thus decreasing overall sensitivity. In a second model, heterodimerization of the shorter variants with the longer variants could impede trafficking to

the cell surface, thereby decreasing the pool of available receptors for ligand binding, which would likewise decrease overall cellular sensitivity. When co-expressed in cultured cells with their predominant full-length receptor forms, truncated variants of some mammalian receptors have been reported to exert dominant negative effects on signaling (Seck et al. 2005; Zmijewski and Slominski 2009; Chow et al. 2012). Alternatively, because many receptor variants exhibit distinct spatial and temporal expression profiles as well as altered ligand binding, atypical feedback regulation, and differential activation of downstream effector pathways (Markovic and Challiss 2009), the multiple PBANR transcripts may reflect a spatio-temporal dependence of functionality. This hypothesis is especially attractive given the pleiotropic complexity of PBAN, the multiplicity of reports detailing PBANR activation by multiple FxPRL-NH_2 peptides (Choi et al. 2003; Watanabe et al. 2007; Kim et al. 2008; Hariton-Shalev et al. 2013; Shalev and Altstein 2015), and the varied expression profile of PBANR transcripts, which have been amplified from diverse tissues including the PG, brain, SOG, ventral nerve cord, thoracic ganglion, ovary, and male abdominal tip (Rafaeli et al. 2007; Watanabe et al. 2007; Bober and Rafaeli 2010; Cheng et al. 2010). Indeed, PBANR expression in larval tissues (Zheng et al. 2007; Kim et al. 2008) suggested possible roles in melanization and/or pupal diapause. Recent studies seem to support this hypothesis with larval-derived and PG-derived PBANRs differing markedly in their three-dimensional conformations, regions/degrees of electrostatic potential, and ligand binding properties (Hariton-Shalev et al. 2013; Shalev and Altstein 2015). While suggestive, these findings require further validation using alternative expression systems, the inclusion of more PBANRs, and the use of various potential endogenous ligands.

2.6 Other FxPRL-NH_2 Receptors

Although significant progress has been made in molecular characterization of PBANRs, the presence of transcripts in diverse tissues, pleiotropic activation (i.e. DH, PBAN, and SGNPs), and the concomitant expression of multiple variants have collectively raised questions regarding the spatio-temporal interactions between the receptor and the FxPRL-NH_2 peptides that regulate pheromonogenesis. These questions were both clarified (and further obscured) following identification of the *B. mori* DH receptor (BommoDHR) (Homma et al. 2006). DH is one of the five FxPRL-NH_2 peptides encoded on the PBAN prepropeptide gene and functions in induction of embryonic diapause and seasonal polyphenism (Imai et al. 1991; Uehara et al. 2011), the termination of pupal diapause in heliothine moths (Xu and Denlinger 2003; Zhang et al. 2004b; Zhao et al. 2004), and prothoracic gland ecdysteroidogenesis (Zhang et al. 2004c; Watanabe et al. 2007). Although BommoDHR was cloned from developing ovaries using a homology-based approach similar to that used for the PBANRs, sequence identity between BommoDHR and the BommoPBANR variants is only ~40% (Homma et al. 2006). DHRs have since been either cloned or identified based on sequence homology from a number of

lepidopterans (Jurenka and Nusawardani 2011). The two receptor types, along with homologs in other insect orders referred to as pyrokinin 1 receptor (PKR1; DHR-like) and pyrokinin 2 receptor (PKR2; PBANR-like), are phylogenetically distinct (Jurenka and Nusawardani 2011; Nusawardani et al. 2013; Jiang et al. 2014). Despite these differences, the activities of DH and PBAN on HelzePBANR and BommoPBANR were reported to be comparable (Choi et al. 2003, 2007; Watanabe et al. 2007). Conversely, PBAN had >20-fold lower activity on BommoDHR (Homma et al. 2006) and no activity on OstnuDHR (Nusawardani et al. 2013), suggesting that greater ligand discrimination occurs with DHR than PBANR. However, functional analyses performed by other groups using different expression systems and assays, came to different conclusions as DH had 15-fold lower activity than PBAN on HelviPBANR-C (Kim et al. 2008) and PBAN activity on HelzeDHR was virtually indistinguishable from DH (Jiang et al. 2014). While these discrepancies likely reflect methodological variances and/or complications associated with heterologous expression (Zhang et al. 2014a), in vitro differences in the efficacy of the two peptides (Stern et al. 2007; Watanabe et al. 2007; Hariton-Shalev et al. 2013; Shalev and Altstein 2015) support regulation of distinct functionalities by the respective ligand-receptor pairs. However, the reduction in pheromonogenesis observed in response to RNA interference (RNAi)-mediated knockdown of PBANRs in *B. mori* (Ohnishi et al. 2006), *P. xylostella* (Lee et al. 2011), and male *H. armigera* (Bober and Rafaeli 2010) have provided unequivocal demonstration of PBANR involvement in mediating the biological effects of PBAN. In those studies, pheromonogenesis was only partially inhibited (~50% reduction) not abolished, suggesting limited penetrance of the dsRNA into the PG cells or that receptor levels, while reduced, were still sufficient to propagate the pheromonogenic signal. Alternatively, those findings may indicate that a full pheromonogenic effect depends on additional endocrine signals and/or other FxPRL-NH_2 receptor/ligand pairs. Despite increasing the complexity of our model for pheromone regulation, the latter hypothesis is attractive as transcripts for both PBANR and DHR have been amplified from PG cDNAs (Watanabe et al. 2007; Nusawardani et al. 2013).

2.7 Structure-Function Analysis of PBANR

2.7.1 Elucidating GPCR Structural Requirements Critical to Ligand Binding and Activation

Targeted disruption of insect neuropeptide signaling, which modulates virtually all aspects of insect biology, physiology and behavior, has been proposed as a novel pest control strategy with great potential for development by the agro-chemical industry (Altstein and Nässel 2010; Audsley and Down 2015). Successful exploitation of this strategy, however, requires a comprehensive understanding of the molecular mechanisms underlying ligand binding and receptor activation. Efforts to determine the atomic structures of GPCRs by standard NMR and X-ray

crystallography methods were initially hampered by the necessity of a lipid bilayer suspension. Consequently, researchers turned to in silico methods using structurally related templates and/or structure-function analyses of GPCR mutants to gain insights into GPCR functionality. Chimeric receptors that incorporate domains from distant, but related GPCRs have also provided insights into the molecular determinants that govern ligand-receptor interactions (Yin et al. 2004) and revealed roles for the N terminus and extracellular loops (ECL) in ligand binding/discrimination (Peeters et al. 2011; also see Chap. 4 volume 2).

2.7.2 PBANR Extracellular Domains

To elucidate the structural determinants governing PBANR activation, Choi et al. (2007) generated a series of chimeric GPCRs that swapped the extracellular domains of HelzePBANR and the *D. melanogaster* pyrokinin receptor 1 (DromePKR1; analogous to DHR), which is ~100-fold less responsive to PBAN. Ligand discrimination was found to largely reside in ECL3, and to a lesser extent the N terminus (Choi et al. 2007), two domains that have been implicated in peptide ligand-GPCR interactions (Gether 2000; Gether et al. 2002; Peeters et al. 2011). Impaired activity following a swap of the respective ECL2 domains was attributed to disruption of the disulfide linkage connecting ECL2 and TM3 that is critical for GPCR folding and ligand binding (de Graaf et al. 2008). To further explore the role of HelzePBANR ECL3 in ligand discrimination, three separate point mutations were later made to residues (G297, S300, and F303) with functional groups that could potentially interact with a peptide ligand (Fig. 8.4a). Alanine substitution of S300 and F303 reduced the efficiency of Ca^{2+} mobilization compared to non-mutated controls in response to PBAN stimulation, suggesting that both residues may comprise potential contact points or contribute to the overall stabilization of the ligand binding pocket (Choi and Jurenka 2010). The role of *N*-glycosylation, which has been linked with efficient cell surface trafficking (Duvernay et al. 2005), was also examined within the context of HelzePBANR-mediated Ca^{2+} influx (Choi et al. 2007). Glutamine substitution of two consensus *N*-glycosylation sites (N19 and N22) in the HelzePBANR N terminus (Fig. 8.4a) negatively impacted PBAN-stimulated Ca^{2+} influx, an effect that was attributed to disruption of forces stabilizing the overall HelzePBANR structure (Choi et al. 2007). However, it is unclear what kind of effect, if any, the substitutions had on receptor trafficking. Deletion of the first 27 residues from the BommoPBANR N terminus, which likewise has two consensus *N*-glycosylation sites (N18 and N21), had no effect on receptor trafficking, ligand binding, or ligand-induced internalization (Hull et al. 2011). This variation in responses may be an artifact of the different assays used to assess functionality, or could reflect intrinsic differences between the respective receptors as *N*-glycosylation effects on GPCR trafficking and activity have been reported to be receptor-dependent (Duvernay et al. 2005).

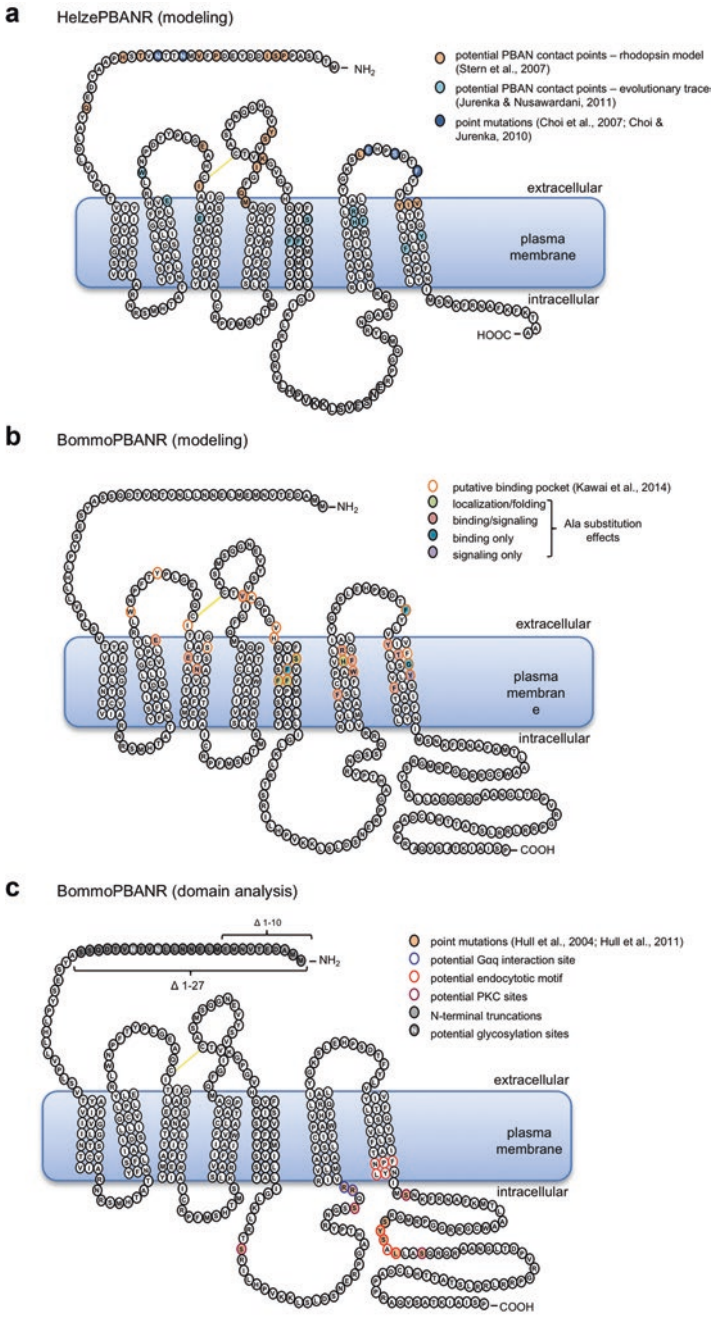

Fig. 8.4 Schematic illustration of sites in *Helicoverpa zea* and *Bombyx mori* PBANRs. (**a**) Residues predicted to comprise the PBAN ligand-binding pocket in HelzePBANR. (**b**) Residues predicted to comprise the PBAN ligand-binding pocket in BommoPBANR. (**c**) Schematic illustration of sites in BommoPBANR-C that have undergone functional analysis via site-directed mutagenesis

2.7.3 HelzePBANR Ligand Pocket

Although rhodopsin (a light sensitive GPCR) is an imperfect template for modeling peptide GPCRs (Sabio et al. 2008; Mobarec et al. 2009; Congreve et al. 2011) it can offer structural insights into potential regions of ligand contact (Congreve et al. 2011). Using molecular docking techniques with a PBAN analog (YFSPRL-NH_2) and a sequence optimized HelzePBANR conformation that utilized coordinates from the bovine rhodopsin crystal X-ray structure, Stern et al. (2007) identified twenty amino acid residues that potentially comprise the ligand binding pocket (Fig. 8.4a). This in silico HelzePBANR structure was also used to evaluate the conformational effects of the ECL swaps (see Sect. 2.7.2) between HelzePBANR and DromePKR1 (Choi et al. 2007). In that evaluation, each domain swap reduced the number of putative ligand contact points. The largest reduction was observed with the ECL2 swap, an effect that likely resulted from misorientation of the cysteines composing the ECL2-TM3 disulfide bridge (Choi et al. 2007). In a complementary approach, Jurenka and Nusawardani (2011) used an evolutionary trace method that mapped conserved residues to a three-dimensional model of HelzePBANR to identify sites critical for ligand selection and binding. The authors of that study further refined their predictions based on the presupposition that the spatial coordinates of GPCR binding domains are frequently evolutionarily conserved. Overall, they identified eleven TM residues potentially comprising a conserved FxPRL-NH_2 binding domain (Fig. 8.4a). They also suggested that the charged residues in HelzePBANR ECL3 (K294, E297, and D301) could potentially contribute to the ligand specificity revealed in the ECL3 domain swap between HelzePBANR and DromePKR1 (Choi et al. 2007), which is consistent with previous reports that ECL3 is involved in ligand specific conformational changes (Gether 2000; Gether et al. 2002; Peeters et al. 2011). However, mutagenesis analysis of E297 had little effect on receptor activity (Choi and Jurenka 2010). Because of the different in silico approaches, the two HelzePBANR models that were developed yielded different aspects of the potential binding pocket. The structural approach focused on the potential role of the ECLs, whereas the evolutionary trace approach focused on identifying the conserved GPCR binding pocket bounded by the TM helices. Taken together, the approaches identified a number of potential ligand interaction points that still await experimental verification.

2.7.4 BommoPBANR Ligand Pocket

In a separate in silico study (Kawai et al. 2014), coordinates based on crystal structures for two class A GPCRS (human β2 adrenergic receptor and human A_{2A} adrenergic receptor) facilitated identification of twenty-seven potential ligand interaction sites in BommoPBANR (Fig. 8.4b). Only three of the twenty potential residues implicated in the rhodopsin-based HelzePBANR structure (Stern et al. 2007) were identified in the BommoPBANR model. However, all of the contact points predicted by the evolutionary trace method (Jurenka and Nusawardani 2011) were

present. Sequential Ala-substitution of the residues revealed roles in ligand binding, receptor activation (i.e. mobilization of extracellular Ca^{2+}), and cell surface trafficking/protein stability. Given their interhelical localization, the four residues (S207, F211, F212, and H284) that affected cell surface expression are predicted to contribute to stabilization of the TM helical bundle. Consequently, the impaired expression observed by the authors was likely the result of receptor misfolding. Kawai and co-workers (2014) further reported a reduction in both ligand binding and receptor activation following Ala-substitution of eleven residues (E95, E120, N124, V195, F276, W280, F283, R287, Y307, T311 and F319), whereas three residues (F209, F303, G315) were implicated in ligand binding alone, and a single residue (Y318) in receptor activation. In this last case, Ala-substitution generated a mutant that exhibited normal ligand binding but impaired receptor activation, suggesting that it may be crucial in the PBAN-induced conformational change that converts the receptor from the non-activated to the activated state. Furthermore, the defects observed with five of the putative binding sites (F212, F276, W280, F283, and F319) may not be exclusively related to ligand binding as they are highly conserved in class A GPCRs and may function in the receptor conformational switch (Holst et al. 2010).

Molecular docking simulations using the BommoPBANR structure and a 5-aa FSPRL-NH_2 analog identified a number of receptor-ligand interactions largely localized to the TM bundle (Kawai et al. 2014). Similar simulations using a NmUR model and a 5-aa analog of NmU further revealed that points of contact between the critical Leu and amide in the respective ligands and the putative binding pockets were conserved: PBANR E95/NmUR E117 (TM2), PBANR E120/NmUR E142 (TM3), PBANR F283/NmUR F313 (TM6), PBANR Y318/NmUR F345 (TM7), and PBANR F319/NmUR Y346 (TM7). The Glu residues in TM2 and TM3 appear to be critically important for ligand binding among the NmUR clade of receptors, as conservation of those sites in other class A GPCRs is more limited (Kawai et al. 2014).

While the ligand-binding pocket described by Kawai and co-workers (2014) is sufficient to accommodate the C-terminal FSPRL-NH_2 active core, steric hindrance precludes it from accepting the full-length 33-aa peptide, suggesting that the non-essential N-terminal portion of PBAN interacts with the ECLs. These interactions could potentially contribute to the stabilization of ligand binding as well as serve as a selectivity filter for differentiating between ligands with similar active cores (i.e. PBAN *vs* DH). In support of this model, two ECL residues (V195 in ECL2 and F303 in ECL3) important in binding the 10-aa PBAN analog were not identified as contact points for the 5-aa analog (Kawai et al. 2014). Furthermore, FxPRL-NH_2 ligand discrimination has been demonstrated experimentally (Homma et al. 2006; Stern et al. 2007; Watanabe et al. 2007; Hariton-Shalev et al. 2013; Nusawardani et al. 2013; Shalev and Altstein 2015) and when functionally important residues in BommoPBANR and BommoDHR are compared, all are conserved with the exception of V195 (Glu in DHR) and F303 (Pro in DHR).

2.7.5 PBANR Intracellular Domains

In contrast to the ligand binding functions of the ECL domains, the C terminus and intracellular loops (ICLs) are critical for propagation and termination of the ligand signal. Ligand binding promotes G-protein dissociation, activation of downstream signal transduction cascades, and subsequent negative feedback regulation/desensitization of the activated GPCR, typically effectuated via endocytotic removal of the receptor from the cell surface (Ferguson 2001; Kristiansen 2004). Knowledge of the specific structural motifs within GPCRs (insect GPCRs in particular) that mediate these processes, however, is limited. Structure-function studies have begun to address this deficiency by providing insights into the mechanisms underlying propagation of the PBAN signal.

2.7.6 G Protein-Coupling

Pheromonogenesis is dependent on an influx of extracellular Ca^{2+} (Jurenka et al. 1991a; Choi and Jurenka, 2004, 2006; Rafaeli 2009; see Sect. 3.2.1). In *B. mori*, this event is mediated by receptor dissociation of a Gαq heterotrimeric G protein (Hull et al. 2010). Receptor-G protein coupling frequently involves ionic interactions between cationic residues near TM6 of the receptor and anionic residues in the C terminus of the G protein (Yang et al. 2002; Kleinau et al. 2010). Alignment of PBANRs with other NmU-clade GPCRs revealed a dibasic site (R263 and R264 in BommoPBANR, see Fig. 8.4c) at this junction (Hull et al. 2011). Ligand-induced internalization, a cellular event that occurs downstream of receptor activation, was significantly reduced following site-directed mutagenesis of these residues (Hull et al. 2011). The disruption in internalization suggests that PBANR signaling was impacted, providing indirect evidence for this region in PBANR-G protein coupling.

2.7.7 C-Terminal Motifs Critical for Ligand-Induced Internalization

A number of conserved C-terminal motifs play critical roles in GPCR desensitization and endocytosis (Ferguson 2001; Kristiansen 2004). The C-terminal region of BommoPBANR has two such motifs, NPxxY (residues 325–329) and YxxΦ (residues 360–363) (Fig. 8.4c). Although NPxxY has been reported to function in the internalization of multiple vertebrate GPCRs (Barak et al. 1995; Gripentrog et al. 2000; He et al. 2001; Bouley et al. 2003), its role in endocytosis is receptor dependent (Slice et al. 1994; Hunyady et al. 1995). The YxxΦ motif (Y = Tyr, x = any amino acid, and Φ = amino acid with a bulky hydrophobic sidechain) has also been implicated in ligand-induced internalization (Paing et al. 2004; Pandey 2009) and is present in the C terminus of numerous peptide GPCRs. Using a series of C-terminal truncations, the BommoPBANR internalization motif was mapped to a 10-aa region spanning residues 357–367, which contain the YxxΦ motif (Hull et al. 2005).

Ala-substitution of the critical residues in the motif likewise impaired internalization (Hull et al. 2005), albeit not to the same extent as the C-terminal truncation, which suggests that, similar to other receptors (Johnson et al. 1990; Nussenzveig et al. 1993; Thomas et al. 1995), the PBANR endocytotic mechanism utilizes multiple signals. The C-terminal YxxΦ motif, YSAL, is highly conserved among the lepidopteran PBANRs and a number of related receptors (i.e. PKR2) in other species, but has diverged in DHRs (YTAM/V), and is not readily apparent in PKR1. This variance suggests that regulation of those receptors either utilizes a different internalization signal or proceeds via a non-endocytotic pathway. Whether or not this sequence is sufficient in and of itself to promote internalization of PBANRs from other species has yet to be experimentally determined.

2.7.8 Phosphorylation-Dependent Internalization of BommoPBANR

Desensitization and internalization of GPCRs are triggered in response to ligand-induced phosphorylation of sites in the ICLs and/or C terminus by G protein-coupled receptor kinases (GRKs) and/or second messenger-dependent kinases, such as protein kinase C (PKC) (Ferguson 2001; Kristiansen 2004). Consistent with this paradigm, BommoPBANR internalization was blocked by the general kinase inhibitor staurosporine (Hull et al. 2005) and significantly impaired following double Ala-substitution of two consensus PKC sites in the BommoPBANR C terminus, S333 and S366 (Hull et al. 2011). In support of PKC-mediated phosphorylation as an internalization trigger, RNAi knockdown of endogenous PKC in Sf9 insect cells also blocked PBANR internalization (Hull et al. 2011). Furthermore, localization of S366 within the 10-aa region (i.e. residues 357–367) critical for ligand-induced internalization (Fig. 8.4c) and the incomplete blockage of internalization following Ala-substitution of the YxxΦ motif are consistent with S366 functioning as a pivotal site for PBANR internalization. Sequence alignments have shown that both the S333 and S366 PKC sites are highly conserved in other PBANRs, which may indicate that feedback regulation of this class of receptors is evolutionarily conserved. Although PKC sites are predicted in the C terminus of most DHRs, the S366 site has not been conserved, providing additional evidence that DHR regulation may proceed via a different pathway.

3 PBAN Signal Transduction

The driving element of numerous studies over the years has been to elucidate the molecular basis underlying conversion of the external PBAN signal into the biological response of pheromone production and release. Initial studies sought to unravel the complex signaling interconnections by examining the effects of various pharmacological compounds (both inhibitors and activators) on pheromonogenesis. While data generated using these compounds can be ambiguous given the possibility of non-specific pharmacological effects and target specificity that varies with

concentration (e.g. NaF at 10 mM acts as a phosphatase inhibitor but at 1–2 mM acts as a G protein activator), it can provide insights into potential mechanisms. Advances in molecular techniques, in particular the applicability of RNAi, have provided additional tools to decipher the molecular components underlying the PBAN signaling cascade. This cascade, which has been most extensively elucidated in heliothine moths (*H. zea*, *H. virescens*, and *H. armigera*) as well as *B. mori*, is now thought to diverge depending on the step in the biosynthetic pathway that is ultimately activated (i.e. early step *vs* late step).

3.1 G Protein Activation

The initial step in most extracellular signal transduction cascades requires dissociation of heterotrimeric guanine nucleotide binding proteins (i.e. G proteins α, β, and γ) from cell surface receptors and subsequent activation of downstream effectors (Cabrera-Vera et al. 2003). Receptor association/dissociation is dependent on the guanine nucleotide binding and hydrolysis activity of Gα subunits, which have been classified based on sequence variation and effector pathways activated into five subtypes: Gαs (stimulate cAMP production), Gαi/o (inhibit cAMP production), Gαq (stimulate Ca^{2+} influx), $G\alpha_t$ (phototransduction), and $G\alpha_{12/13}$ (actin cytoskeletal remodeling) (Cabrera-Vera et al. 2003; Meigs and Lyakhovich 2012). Prior to PBANR identification, PBAN-induced elevation of cAMP levels (Rafaeli and Soroker 1989; also see Sect. 3.3.1) and the pheromonotropic effects of NaF (1–2 mM) on isolated PGs (Rafaeli and Gileadi 1996a) suggested the involvement of G proteins in the PBAN signal transduction cascade. Using homology-based cloning and genomic mining methods, transcripts for four Gα subunits (two Gαs, a Gαo, and a Gαq) were amplified from *B. mori* PGs (Hull et al. 2007a, 2010). Sequential RNAi knockdown of the four Gα subunits revealed that only Gαq had a role in transmitting the PBAN pheromonotropic signal (Hull et al. 2010).

3.2 PBAN-Induced Influx of Ca^{2+}

3.2.1 Essential Role of Extracellular Ca^{2+}

Initial studies using isolated PGs from diverse moth species demonstrated that the pheromonotropic effects of PBAN require extracellular Ca^{2+} (Jurenka et al. 1991a; Fónagy et al. 1992d; Jurenka et al. 1994; Ma and Roelofs 1995; Matsumoto et al. 1995b; Soroker and Rafaeli 1995; Zhao et al. 2002; Choi and Jurenka 2004, 2006; Hull et al. 2007a). Moreover, pharmacological manipulation (e.g. ionomycin, A23187, or thapsigargin) of intracellular Ca^{2+} levels could trigger pheromone production (Jurenka et al. 1991a; Fónagy et al. 1992c, d; Rafaeli 1994; Jurenka et al. 1994; Ma and Roelofs 1995; Matsumoto et al. 1995a, b; Soroker and Rafaeli 1995;

Rafaeli and Gileadi 1996a; Zhao et al. 2002; Hull et al. 2007a), whereas inorganic Ca^{2+} channel blockers inhibited pheromone production (Jurenka et al. 1991a; Fónagy et al. 1992d; Ma and Roelofs 1995; Matsumoto et al. 1995b; Choi and Jurenka 2004). Taken together, these findings provided indirect evidence for PBAN-dependent opening of cell surface ion channels and the concomitant influx of Ca^{2+}. Subsequent advances in fluorescent Ca^{2+} imaging techniques provided direct evidence for the rise in intracellular Ca^{2+} in response to PBAN binding in isolated *H. zea* and *B. mori* PGs (Choi and Jurenka 2006; Hull et al. 2007a).

3.2.2 Identification of the PBAN-Activated Ca^{2+} Channels

The most pervasive Ca^{2+}-permeable ion channels in cells are voltage-operated channels (VOCs) (Lacinova 2005) and receptor-activated Ca^{2+} channels (RACCs) (Prakriya and Lewis 2015; Redondo and Rosado 2015), which include diacylglycerol (DAG)-dependent channels and store-operated channels (SOCs). Consistent with the early prediction of receptor involvement, VOC blockers had no effect on pheromone production in *H. zea* (Jurenka et al. 1991a; Choi and Jurenka 2006) or *B. mori* (Hull et al. 2007a), whereas SKF-96365, an inhibitor of both VOC and RACC, had pronounced pheromonostatic effects in *H. virescens* and *B. mori* (Jurenka 1996; Hull et al. 2007a). Further pharmacological manipulation of channel activity using inhibitors/activators of SOCs suggested that PBAN signals through an SOC pathway rather than a DAG-dependent channel (Hull et al. 2007a).

For many systems, the SOC pathway consists of stromal interaction molecule 1 (STIM1) functioning as a Ca^{2+} sensor and Orai1 as the pore-forming unit of the channel (López et al. 2016). Consistent with a role in the PBAN-activated SOC pathway, targeted knockdown of *B. mori* homologs of STIM1 and Orai1 negatively affected pheromone production without affecting non-pheromonotropic enzyme activities (Hull et al. 2009). The dependence on extracellular Ca^{2+} in PBAN-regulated pheromone pathways and the presence of STIM1 and Orai1 transcripts in moth PG transcriptomes (Ding and Löfstedt 2015) suggests that the STIM1-Orai1 SOC pathway is likely conserved in moths.

3.3 Role of Other Second Messengers

3.3.1 cAMP

While extracellular Ca^{2+} has been shown to be an absolute requirement for pheromonotropic activity in every moth species studied to date, the role of cAMP in the PBAN signal cascade appears to be species-dependent. Early cAMP radioimmunoassays demonstrated a PBAN-mediated increase of cAMP levels in isolated *H. armigera* PGs (Rafaeli and Soroker 1989; Rafaeli 1994; Soroker and Rafaeli 1995; Rafaeli and Gileadi 1996a). Furthermore, pharmacological manipulation (e.g.

cAMP analogs, phosphodiesterase inhibition, or adenylate cyclase activation) of PG cAMP levels promoted pheromone production in *H. armigera* (Rafaeli and Soroker 1989; Soroker and Rafaeli 1995; Rafaeli and Gileadi 1996a), *H. zea* (Jurenka et al. 1991a), *H. virescens* (Jurenka 1996), and *Argyrotaenia velutinana* (Jurenka et al. 1994). In contrast, similar studies failed to find cAMP-linked pheromonotropic effects in *B. mori* (Fónagy et al. 1992d), *S. litura* (Matsumoto et al. 1995b), or *O. nubilalis* (Ma and Roelofs 1995) and no evidence was found of PBAN-mediated cAMP elevation in *B. mori* PGs (Hull et al. 2007b). There is, however, a strong correlation between this second messenger event and the pheromone biosynthetic activity under PBAN control. In species that utilize cAMP, the pheromonotropic control point resides in fatty acid biosynthesis, most likely the acetyl-CoA carboxylase (Tang et al. 1989; Jurenka et al. 1991b; Tsfadia et al. 2008). However, in species that do not undergo cAMP elevation, PBAN regulates a step(s) further along in the biosynthetic pathway, usually fatty acyl reduction (Fabriàs et al. 1994; Ma and Roelofs 1995; Ozawa et al. 1995; Ozawa and Matsumoto 1996; Moto et al. 2003; Eltahlawy et al. 2007) and, *in B. mori*, a second step involving cytoplasmic lipid droplet lipolysis (Fónagy et al. 2000; Ohnishi et al. 2006). While the evidence is currently too limited to draw broad conclusions regarding the relationship between cAMP signaling and PBAN regulation, the predictable associations suggest an avenue of potential research, in particular within species (*Thaumetopoea pityocampa*, *M. sexta*, *Sesamia nonagrioides*) in which the pheromonotropic control point is known be a step late in biosynthesis (Fabriàs et al. 1995; Fang et al. 1995; Mas et al. 2000) or species (*Ostrinia furnacalis*, *M. brassicae*, *Dendrolimus punctatus*, *P. separata*) where PBAN regulates a step in the fatty acid pathway (Jacquin et al. 1994; Zhao and Li 1996; Zhao et al. 2002; Fónagy et al. 2011; Köblös et al. 2015). It would likewise be interesting to examine the role of the PBANR variants in the contrasting signal transduction cascades. Jurenka and Rafaeli (2011) proposed that structural variations in the C-terminal lengths of the PBANR variants may contribute to the differing downstream responses with shorter C-terminal tail PBANRs linked to cAMP dependent pathways and the longer C-terminal PBANRs linked to Ca^{2+} influx alone.

3.3.2 IP_3

Similar to Ca^{2+} and cAMP, the phosphoinositide IP_3 (inositol 1, 4, 5-triphosphate) is a signal transduction messenger. IP_3 is generated from phospholipase C (PLC)-mediated hydrolysis of PIP_2 (phosphatidylinositol-4,5-bisphosphate) in response to receptor activation and typically functions in the propagation of receptor-mediated Ca^{2+} signaling by mobilizing intracellular Ca^{2+} stores (Balakrishnan et al. 2015). An early study on the PBAN mode of action reported that pheromonotropic activity of *H. armigera* PGs was reduced following pharmacological depletion of IP_3 (Rafaeli 1994). A later study in *B. mori* reported that total inositol phosphate levels in isolated PGs rose in response to PBAN and that RNAi knockdown of a putative IP_3 receptor suppressed pheromone production (Hull et al. 2010). These findings

implicated PBANR-mediated activation of PLC. In support of this, pharmacological inhibition of PLC activity with either U73122 or compound 48/80 negatively impacted pheromone production in *B. mori*, whereas the inactive analog of U73122 had no effect (Hull et al. 2010). The pheromonostatic effects of compound 48/80, however, differed from a previous study that found no effect on *B. mori* pheromone production (Matsumoto et al. 1995a). Given that the preponderance of evidence available with the more recent study strongly pointed to PLC activity, the contrasting result was attributed to methodological differences. Separate studies demonstrating the critical importance of SOC components STIM1 and Orai1 (see Sect. 3.2.2 and Hull et al. 2009) in pheromone production likewise implicated PLC activity.

3.4 PBAN-Mediated PLC Activity

PCL-dependent activation of SOCs is predominantly driven by PLCβ and PLCγ (Drin and Scarlata 2007). PLCβ is generally activated downstream of GPCRs (Drin and Scarlata 2007), whereas PLCγ functions downstream of tyrosine kinase and non-receptor tyrosine kinases (Patterson et al. 2005). Using genomic mining methods, PLCβ1, PLCβ4, and PLCγ transcripts were amplified from *B. mori* PGs (Hull et al. 2010). Consistent with the expected signaling paradigm, RNAi-mediated knockdown of PLCβ1 significantly reduced pheromone production. PLCγ knockdown likewise mitigated the pheromonotropic effects of PBAN (Hull et al. 2010). Based on findings in other systems (Patterson et al. 2005), PLCγ was postulated to function in PBAN signaling as a molecular scaffold that stabilizes the protein-protein interactions essential for formation of the SOC complex rather than catalyzing PIP_2 hydrolysis.

3.5 Signal Transduction Post-PBAN-Mediated Ca^{2+} Influx

3.5.1 Calmodulin

As discussed above, the role of cAMP in PBAN signaling appears to differentiate the enzymatic step in the respective sex pheromone biosynthetic pathways under PBAN control. The GPCR-mediated generation of cAMP can be an indication that the receptor couples through Gαs, which stimulates adenylate cyclase activity following receptor dissociation. However, cAMP production in *H. armigera* reportedly occurred downstream of Ca^{2+} influx (Soroker and Rafaeli 1995), suggesting the involvement of a Ca^{2+}-dependent adenylate cyclase. Additional pharmacological profiling of the PBAN cascade revealed that inhibition of calmodulin, a multifunctional Ca^{2+} binding protein that interacts with diverse proteins, blocked the PBAN-mediated increase of cAMP in *H. armigera* (Rafaeli and Gileadi 1996a) and

mitigated the pheromonotropic effects of PBAN in *H. armigera* (Soroker and Rafaeli 1995) as well as *S. litura* and *B. mori* (Matsumoto et al. 1995a, b; Ozawa and Matsumoto 1996). In support of these results, a calmodulin homolog identical to the *D. melanogaster* protein was purified from *B. mori* PGs (Iwanaga et al. 1998). Among the enzymatic activities reportedly mediated by Ca^{2+}-bound calmodulin are adenylate cyclases (Halls and Cooper 2011), suggesting that the Ca^{2+}-dependent increase in cAMP observed in heliothine moths is likely driven by one of these cyclases. Because many calmodulin interacting proteins are directly or indirectly involved in protein phosphorylation, the results observed in *S. litura* and *B. mori*, neither of which utilizes cAMP in PBAN signaling, may be attributable to impaired phosphorylation cascades.

3.5.2 Kinase Activity

GPCR-mediated activation of biosynthetic pathway enzymes typically involves a phosphorylation cascade driven by diverse kinase (phosphorylation) and phosphatase (dephosphorylation) steps. The generation of cAMP, the critical role of calmodulin, and the importance of PKC in feedback regulation of BommoPBANR in vitro (see Sect. 2.7.8) strongly suggested kinase activity in PBAN signaling. While early studies assessing the effect of both broad spectrum and specific kinase inhibitors found no effect on pheromone production in either *B. mori* (Matsumoto et al. 1995a) or *H. armigera* (Soroker and Rafaeli 1995), the PKC activator, phorbol 12-myrstate 13-acetate (PMA), was found to have pheromonotropic activity in *H. armigera* (Soroker and Rafaeli 1995). This effect, however, did not extend to *B. mori* or *S. litura* (Matsumoto et al. 1995b; Ozawa et al. 1995). A more recent study using anti-phosphoamino acid antibodies found clear evidence of PBAN-mediated phosphorylation in *B. mori* (Ohnishi et al. 2011). Furthermore, RNAi-mediated knockdown of a Ca^{2+}-bound calmodulin dependent kinase II (CaMKII) in *B. mori* PGs reduced PBAN-induced pheromone production and diminished phosphorylation of a critical lipid droplet-associated protein, whereas knockdown of putative protein kinase A (PKA) and PKC transcripts had no effect (Ohnishi et al. 2011).

3.5.3 Phosphatase Activity

In contrast to the early kinase inhibitor studies, pharmacological inhibition of phosphatase activity had pronounced pheromonostatic effects in *B. mori* (Matsumoto et al. 1995a, b; Ozawa and Matsumoto 1996; Fónagy et al. 1999) as well as *H. zea* and *H. virescens* (Jurenka 1996). Inhibition of ionophore-induced pheromone production in *H. zea* suggested that phosphatase activity occurs downstream of Ca^{2+} influx (Jurenka 1996), thus ruling out an effect similar to LiCl, which inhibits IP_3 generation. The effectiveness of inhibitors specific for calcineurin (Fónagy et al.

1999), a protein phosphatase b activated by Ca^{2+}-bound calmodulin, was consistent with previous studies demonstrating calmodulin activity. In support of this role, both calcineurin subunits were amplified from *B. mori* PGs (Yoshiga et al. 2002). Determination of the rate-limiting steps in heliothine moths and *B. mori* suggest that calcineurin or calcineurin-like phosphatase activity comprises the penultimate control point in PBAN signaling. In heliothine moths, PBAN activates acetyl-CoA carboxylase, the critical point in fatty acid biosynthesis that catalyzes carboxylation of acetyl-CoA to yield malonyl-CoA. In *B. mori* (and other moths), PBAN regulates a fatty acyl reductase that shares biochemical characteristics with HMG-CoA reductase (Ozawa et al. 1995). In both cases (i.e. acetyl-CoA carboxylase and HMG-CoA reductase), enzymatic activity is phosphorylation-dependent (Zammit and Easom 1987; Brownsey et al. 2006).

3.6 Model of Pheromone Regulation by PBAN Signaling

Based on diverse studies spanning more than 20 years (many of which were briefly described above), a model for the molecular signaling cascade underlying PBAN-mediated regulation of pheromone production has emerged (Fig. 8.5). Circadian activation of extero-receptors and brain hormones such as allatotropins/allatostatins that influence JH biosynthesis (Cusson and McNeil 1989; Woodhead et al. 1989; Picimbon et al. 1995; Stay and Tobe 2007) may have a role in PBAN release into the hemolymph where it interacts with PBANRs localized at the plasma membrane of PG cells. The ensuing conformational change in PBANR results in dissociation of the heterotrimeric G protein complex with subsequent Gαq activation of PLCβ1-mediated hydrolysis of PIP2 into DAG and IP_3. The soluble IP_3 diffuses through the cytosol to activate IP_3 receptors in the endoplasmic reticulum (ER) membrane, which promotes release of stored Ca^{2+}. The drop in luminal Ca^{2+} levels results in translocation of STIM1 to the plasma membrane where it triggers an influx of extracellular Ca^{2+} through Orai1 channels, presumably via interactions with a scaffolding complex that includes PLCγ. The concomitant rise in intracellular Ca^{2+} allows for formation of Ca^{2+}-calmodulin complexes, at which point the pathway exhibits species-dependent divergence. In heliothines and species that utilize cAMP, the Ca^{2+}-calmodulin complexes stimulate adenylyl cyclase activity. The rise in cAMP then drives a cascade culminating in activation of the fatty acid biosynthetic pathway enzyme, acetyl CoA-carboxylase. In *B. mori,* and presumably species in which PBAN regulates a step late in pheromonogenesis, the Ca^{2+}-calmodulin complexes activate both calcineurin (a protein phosphatase) and calmodulin-dependent kinase II (CamKII). Calcineurin in turn activates fatty acyl reductase, the terminal step in pheromone biosynthesis, while CamKII-dependent phosphorylation of lipid storage droplet protein-1 promotes lipolytic release of stored pheromone precursors (Fig. 8.5).

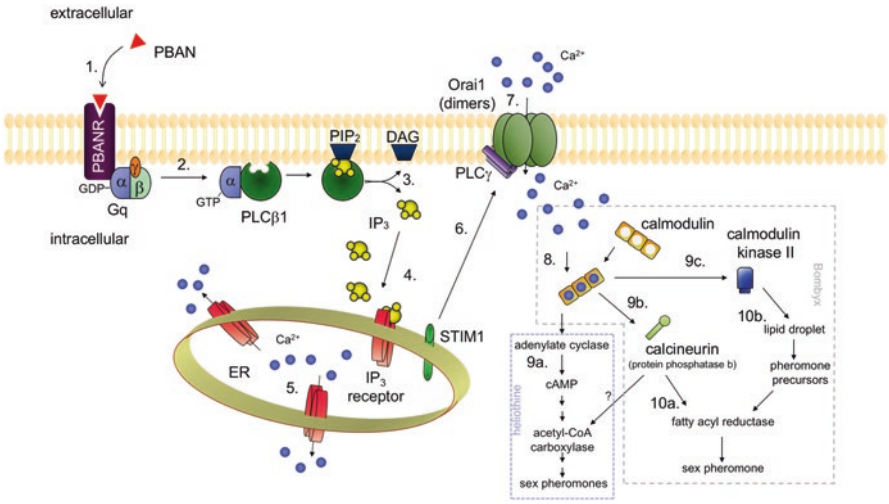

Fig. 8.5 Proposed PBAN signal transduction cascade. (*1*) PBAN circulating in the hemolymph binds to PBANR in the plasma membrane of PG cells. (*2*) PBAN binding promotes dissociation of Gαq from PBANR with subsequent activation of PLCβ1. (*3*) PLC-mediated hydrolysis of PIP_2 yields DAG and soluble IP_3. (*4*) Cytosolic IP_3 interacts with IP_3 receptors in the ER membrane. (*5*) Activation of IP_3 receptors promotes release of stored Ca^{2+}. (*6*) The reduction in ER luminal Ca^{2+} levels promotes interactions between STIM1 and Orai1 channels in the plasma membrane. The resulting complex formation may be stabilized by protein-protein interactions with SH3 domains in PLCγ. (*7*) The activated Orai1 channels open allowing an influx of extracellular Ca^{2+}. (*8*) Free calmodulin complexes with the intracellular Ca^{2+}. (*9a*) In heliothines, the Ca^{2+}-calmodulin complex stimulates adenylate cyclase activity and the production of cAMP, which subsequently initiates a protein kinase A/C phosphorylation cascade. PBAN signaling culminates in activation of acetyl-CoA carboxylase, the limiting step in fatty acid biosynthesis. Given evidence in the literature that this enzyme is activated in response to dephosphorylation and that pharmacological inhibition of phosphatase activity in *H. zea* and *H. virescens* has pheromonostatic effects, it is likely that a protein phosphatase, possibly calcineurin, may function in acetyl-CoA carboxylase activation. (*9b*) In *B. mori*, calcineurin is activated by the Ca^{2+}-calmodulin complex, which also activates (*9c*) a calmodulin-dependent protein kinase II (CamKII). (*10a*) CamKII phosphorylates a lipid droplet storage protein critical for lipolytic release of pheromone precursors stored in cytosolic lipid droplets. (*10b*) Calcineurin dephosphorylates fatty acyl reductase, the terminal enzymatic reaction in the *B. mori* pheromone biosynthetic pathway. Abbreviations: *cAMP* cyclic adenosine 3′, 5′-monophosphate, *DAG* diacylglycerol, *ER* endoplasmic reticulum, *GDP* guanosine diphosphate, *Gq* G protein α subunit q, *GTP* guanosine-5′-triphosphate, *IP_3* inositol 1,4,5-trisphosphate, *PIP2* phosphatidylinositol (4,5)-bisphosphate, *PLC* phospholipase C, *STIM1* stromal interaction molecule 1

4 Targeted Disruption of PBAN Pathway

Current integrated pest management strategies that focus on mating disruption frequently exploit synthetic pheromone blends (Witzgall et al. 2008; El-Sayed et al. 2009). However, for species that utilize multi-component pheromone blends with cost prohibitive chemistries, targeted disruption of pheromone biosynthetic

pathways has significant potential as an alternative control measure. This is the case for the black cutworm moth, *A. ipsilon*, a polyphagous, polyandrous pest with multi-continental populations and intra-specific genetic variations (Wakamura et al. 1986; Picimbon et al. 1995, 1997; Gadenne et al. 1997; Duportets et al. 1998; Gemeno and Haynes 1998; Gemeno et al. 2000; Du et al. 2015). Insect GPCRs in particular have been proposed as promising targets for the next generation of insecticides (Scherkenbeck and Zdobinsky 2009; Van Hiel et al. 2010; Bai and Palli 2013; Grimmelikhuijzen and Hauser 2013; Audsley and Down 2015). This interest has driven significant efforts in developing peptidomimetics that overcome limitations (i.e. environmental instability, poor cuticular penetrance, and susceptibility to proteolytic degradation in the hemolymph) inherent to peptides that make them unsuitable for pest management. Because this topic has been extensively reviewed elsewhere (Altstein 2001, 2004b; Nachman et al. 2009a; Scherkenbeck and Zdobinsky 2009), we provide only a brief overview of some of the most intriguing developments.

4.1 Peptidomimetics

4.1.1 PBAN Agonists

PBAN agonists, small molecules that activate the receptor in the absence of the endogenous ligand, provide valuable insights into the structural requirements and chemistries crucial for ligand binding and cuticular penetration. In addition, they offer possibilities in pest management as continuous pheromonogenic stimulation via a bound agonist could lead to pheromone release asynchronous with male mating behaviors and/or depleted pheromone. Early peptide engineering studies revealed that modification of the terminal Phe in the pentapeptide FTPRL-NH_2 with a hydrophobic cage-like *o*-carborane moiety (a cluster composed of boron, carbon, and hydrogen), 1-pyrenebutyric acid, 9-fluoreneacetic acid, or 2-amino7-bromofluorene yielded topically active pheromonogenic analogs with enhanced cuticular penetrance and greater hemolymph persistence (Nachman et al. 1996; Teal and Nachman 1997, 2002). Additional studies incorporating β-amino acids further highlight the importance of the Phe residue for pheromonotropic activity (Nachman et al. 2009a).

4.1.2 PBAN Antagonists

The structural, conformational and dynamic features of agonists can serve as the basis for rational design of antagonists, which require the compound to bind the receptor without activating the signal transduction cascade. Replacing the Thr in the pheromonogenic septapeptide RYFTPRL-NH_2 with D-Phe yielded a linear peptide antagonist that significantly inhibited pheromone production following injection

(Zeltser et al. 2000). Backbone cyclization techniques have also yielded antagonists with pheromonostatic effects that can persist for several hours (Altstein et al. 2000). A linear RYF[dF]PRL-NH_2 analog that incorporated an aliphatic amine exhibited enhanced cuticular penetration while retaining pheromonostatic properties (Nachman et al. 2009b).

4.1.3 Receptor Selective Analogs

FxPRL-NH_2 analogs have been reported to have differing receptor effects depending on the activity assayed (e.g. melanotropic *vs* pheromonotropic) despite mediation of both activities by the same peptidergic sequence (Matsumoto et al. 1992; Altstein et al. 1996) and receptor (Zheng et al. 2007; Kim et al. 2008). Sequential D-Phe scan of a modified PBAN sequence (YFSPRL-NH_2) generated a selective antagonist that significantly reduced pheromone production with no effect on pupal melanization (Ben-Aziz et al. 2005). An amphiphilic version of the antagonist, that incorporated an aliphatic amine via succinic acid at the N terminus of the pentapeptide, retained selective antagonist properties while exhibiting enhanced cuticular penetrance (Nachman et al. 2009b). Similarly, replacement of the critical Phe with a β-homo-amino acid yielded an analog that affected melanization but had no effect on pheromone production (Nachman et al. 2009a). Incorporation of a dihydroimidazoline moiety into the FxPRL-NH_2 hexapeptide sequence likewise generated a selective melanotropic antagonist devoid of either pheromonotropic or pheromonostatic activities (Nachman et al. 2010). The selectivity observed in these peptidomimetic studies suggests that the melanotropic receptor tolerates greater conformational deviations in the ligand than the pheromonotropic receptor. This ligand selectivity is corroborated by both in vitro and in silico studies of FxPRL-NH_2 receptors that show dissimilar three-dimensional conformations, electrostatic potentials, and ligand preferences (Hariton-Shalev et al. 2013; Shalev and Altstein 2015). While the development of selective antagonists will undoubtedly provide additional insights into the development of novel pest management agents, it is apparent that despite years of study, our understanding of FxPRL-NH_2 pleiotropism at the molecular level will remain a fertile area of research.

4.2 RNAi: The New Frontier?

As a biorational approach that can be specifically tailored to individual pest species, RNAi holds great promise for the future of insect pest management (Price and Gatehouse 2008; Burand and Hunter 2013). Though still in its infancy, the viability of using transgenic plants that trigger RNAi-mediated suppression of select pest genes has been effectively demonstrated (Baum et al. 2007; Mao et al. 2007, 2011; Pitino et al. 2011). While those studies focused on the control potential associated with knockdown of diverse enzymes, current studies assessing the effects of

neuropeptide/GPCR RNAi knockdown on peptidergic regulation of insect biology (e.g. Terhzaz et al. 2007; Arakane et al. 2008; Badisco et al. 2011; Bai et al. 2011; Terhzaz et al. 2015; Zandawala et al. 2015) may provide an additional biorational set of tools for the development of next generation pest management strategies.

4.2.1 RNAi-Knockdown: PBAN

To date, RNAi-mediated knockdown of PBAN has only been reported for two species, *H. zea* (Choi et al. 2012) and *S. litura* (Lu et al. 2015). In both species, injection of double-stranded RNAs (dsRNAs) corresponding to a fragment of the respective DH-PBAN gene markedly reduced sex pheromone production. In *H. zea*, however, the PBAN dsRNA injections, which were performed using 4–5 day old female pupae, also affected adult emergence with a significantly higher percentage of injected pupae unable to eclose (Choi et al. 2012). A similar phenotype was reported in another heliothine moth following knockdown of PBAN, but not PBANR, suggesting that the failure to eclose properly may be linked to DH, which functions in termination of pupal diapause in heliothine moths (Xu and Denlinger 2003; Sun et al. 2003).

4.2.2 Genome Editing: PBAN

Advances in genome editing methodologies have extended targeted gene mutagenesis capabilities. One such approach utilizes Transcription Activator-Like Effector Nucleases (TALENs) to introduce small deletions or insertions at the gene level that cause frameshift mutations/truncations. Recently, Shiomi et al. (2015) used this method to make targeted deletions in the *B. mori* DH-PBAN gene yielding prepropeptides severely truncated within the signal peptide region precluding generation of the PBAN sequence. While the mutations clearly affected the induction of embryonic diapause, the pheromonogenic effects, which were not the focus of the study and were thus only assessed superficially, appeared to be muted with a slight reduction in the male behavioral response.

4.2.3 RNAi-Knockdown: PBANR

PBANR transcripts have been knocked-down in *B. mori* (Ohnishi et al. 2006), *P. xylostella* (Lee et al. 2011), and *H. armigera* (Bober and Rafaeli 2010). Injection of dsRNAs corresponding to a 417-nt fragment of BommoPBANR into 1-day-old pupae triggered receptor knockdown and significantly impaired sex pheromone production and disrupted lipolysis of cytoplasmic lipid droplets (Ohnishi et al. 2006). Similarly, knockdown of PluxyPBANR in pupae 1 day prior to adult emergence with dsRNAs corresponding to a 549-nt fragment resulted in a ~50% reduction in sex pheromone production and a 20–40% reduction in female mating (Lee et al.

2011). That group also reported decreased expression of two desaturases thought to be involved in the *P. xylostella* sex pheromone biosynthetic pathway following PluxyPBANR knockdown (Lee and Kim 2011). Unlike *B. mori* and *P. xylostella*, the effects of HelarPBANR knockdown were evaluated in adult male moths. An earlier study reported expression of HelarPBANR in the male aedeagus, a reproductive organ adjacent to the male abdomen through which sperm from the testis is transferred during copulation and which is usually associated with male-derived sex pheromone-like compounds (Rafaeli et al. 2007). Injection of dsRNAs corresponding to a 880-nt fragment of HelarPBANR in 1-day-old adult male *H. armigera* significantly reduced PBAN-stimulated production of male volatile compounds (Bober and Rafaeli 2010). While the relevance of these compounds in *H. armigera* mating behavior remains to be demonstrated, similar compounds have been linked to stimulation of female receptivity and inhibition of male competition (Teal and Tumlinson 1984; Kehat and Dunkelblum 1990; Huang et al. 1997; Hillier and Vickers 2004; Hillier et al. 2006). In the European corn borer, *O. nubilalis*, the male scent odor is crucial for the acceptance of the male by the female (Royer and McNeil 1992; Picimbon 1996; Farrell and Andow 2017). Regardless, the results demonstrate that in a wide variety of moths the role of PBANR functionality in pheromone biosynthesis is certainly not restricted to females and further underscores the pleiotropic nature of the receptor and its multifunctional ligand.

5 Concluding Remarks

The past 30 years have witnessed significant progress in our understanding of pheromonogenesis in moths and its neuroendocrine regulation. Interestingly, rather than clarifying our understanding of pheromonotropic control, elucidation of the "black box" has illuminated yet another layer of complexity and provided new puzzles for us to unravel.

Some of the questions raised with this new framework of entomology, chemical ecology, physiology and molecular biology research that we find the most intriguing include:

- What is the molecular basis for regulation of the pleiotropic FxPRL-NH_2 peptide/receptor system?
- How is ligand selectivity of PBANRs/DHRs achieved?
- What is the evolutionary significance of the different control points (fatty acid biosynthesis *vs* terminal modification) in the PBAN pathway, and how did this divergence arise?
- What biological role do the concomitantly expressed PBANR variants play in PBAN signaling?
- How are transcription and alternative splicing of PBANR regulated?

Undoubtedly, rapid developments in mRNA sequencing, bioinformatics, molecular engineering, and proteomics will play a significant role in resolving these new

questions. In addition, advances such as CRISPR in insect genome editing (Taning et al. 2017), and RNAi (see Chap. 5), despite the current limitations of this technology in lepidopterans (Terenius et al. 2011), can provide unequivocal demonstration of the roles calmodulin, calcineurin, and acetyl-CoA carboxylase have in heliothine pheromonogenesis and finally reveal how conserved PBAN signaling pathways function across species. Similar application of these technologies can also provide insights into the role of antagonistic peptidomimetics in receptor regulation.

Continued research into the mechanisms underlying PBANR function in moths, as well as related receptors in other species, will help answer questions regarding the biological significance of the FxPRL-NH_2 family and how alternative splicing plays a role in mediating that biology. This knowledge will provide insights into the complexities of GPCRs, and can potentially be applied towards the development of novel biorationally designed insect control agents. These fundamental studies will also continue to provide insights into mammalian endocrinology, lipid biology, and the molecular interactions underlying peptidergic binding/activation of pleiotropic GPCRs.

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Glossary

^{3}H-labeled pheromone A synthetic pheromone with a hydrogen atom exchanged by tritium (^{3}H, the radioactive isotope of hydrogen) used in pionnering biochemical studies in order to measure the pheromone concentration using the beta radiation

Acetylcholine (*Ach*) An organic neurotransmitter chemical that functions in the brain of many organisms including human and insects

Acheta domesticus (house cricket) A study model for neurogenesis in the brain

Actinobacter A genus of Gram-negative bacteria belonging to the class of Grammaproteobacteria known to occur in pairs

Active core (FxPRL-NH_2) A specific motif in the PBAN pentapeptide that is active in inducing pheromone production

Acyrthosiphon pisum (pea aphid) A sap-sucking insect in the Aphididae family

ADAR (adenosine deaminase acting on RNA) An enzyme that recognizes specific RNA duplexes and affects RNA sequence through adenosine (A)-to-Inosine (I) mutations

Aedes aegypti The yellow fever mosquito that is responsible for epidemiological diseases such as Dengue, Chikungunya and Zika

Aggregation pheromone An odor that attracts members of the same species (conspecifics) to the same location for mate selection or defence against predators

Agrotis ipsilon (black cutworm moth) A long-lived migrant species of moth (Noctuidae), polyphagous, widespread, damaging particularly in the Northern hemisphere and known to postpone its activities linked to pheromone production and reproduction

Agrotis segetum (turnip moth) An owlet moth of the family Noctuidae, largely spread particularly in Europe, species of the same genus than *A. ipsilon* with whom interspecific hybrids can be obtained in laboratory conditions

Alarm pheromone A highly volatile odor pheromone chemical used to alert nestmates for danger (predator) and colony defense

Aldehyde oxidase A metabolic enzyme that catalyzes the oxidation of aldehydes into carboxylic acids

J.-F. Picimbon (ed.), *Olfactory Concepts of Insect Control - Alternative to Insecticides*, https://doi.org/10.1007/978-3-030-05060-3

Allelochemical (*allelo* = "another") A chemical produced by an organism that has an effect on individuals of another species when released (e.g. toxic chemicals released by the plants in response to herbivorous attacks)

Alpha-helix A basic structure in the protein characterized by a rod-like spatial configuration

Alternative splicing A regulated process of intron removal during gene expression that results in a single gene coding for multiple protein isoforms

Anopheles gambiae The primary mosquito vector for the transmission of Malaria

Anosmia Inability to sense an odor or a perfume scent

Ant An eusocial (from Greek Ɛŭ = "good") insect that lives in colonies (nests) similarly to bees and wasps with whom they share common ancestry (order Hymenoptera)

Antagonist An organism that competes with another (one species is negatively affected); A drug or chemical that binds to a receptor and blocks (or alters) the biological response by interfering with the interaction to the natural compound at the same receptor site

Antennal lobe The region of the insect brain which receives the input from the antennae

Antheraea polyphemus, A. pernyi A giant silkmoth (Saturniid) with large, double-combed male antennae, with one cm^2 outline area and 60,000 sensilla trichodea, each 300 μm long

Antimicrobial Peptide (host defense peptide) A 12–50 amino acids-long peptide with the potency to kill microbes and/or modulate the immune system as part of the innate immune response found among the whole class of life, including insects

Aphrodisiac An odor released by the male to facilitate its acceptance by the female as found in pyralid moths

Apis cerana The common Asiatic or Eastern honey bee

Apis mellifera The common European or Western honey bee

Apoptosis (from ancient Greek àπόπ⎡= "falling off") The process of programmed cell death that occurs in all multicellular organisms

Arbovirus (arthropod-born virus) An informal name in modern medicine to refer to viruses that are transmitted by arthropod vectors of infectious diseases

Arthralgia (arthro = "joint", algos = "pain") A pain in one or more joints symptomatic of epidemiological diseases vehiculated by insects

Arthropod An invertebrate organism having an external skeleton (exoskeleton), a segmented body, and paired jointed appendages (insects, arachnids, myriapods and crustaceans)

Atmosphere (from Greek atmos = "vapour" and sphaira = "sphere") A layer of gases such as argon, carbon dioxide, nitrogen and oxygen surrounding Earth, held in place by the gravity of Earth and maintained if this gravity exerted by Earth is high and the global layer temperature is low enough, among others

Bacillus A genus of gram-positive aerobic, motile rod-shaped bacteria (firmicute, the most heat-resistant organism known on earth)

Bacillus thuringensis A soil-dwelling *Bacillus* bacterium naturally occurring in the gut of caterpillars and commonly used for insect pest control

Base pair mismatch A typo change in the genetic sequence that causes a point mutation

Bemisia tabaci The sweetpotato whitefly, principal threat to green vegetation worldwide

Beta sheet Another major type of conformation (formed by β-strands) observed in protein structures

Bimodality The simultaneous use of two distinct conditions, modalities or systems

Biocontrol The aim of controlling insect pest species using other insects or organisms

Biologically relevant odorant An odor molecule (or chemical signal) that can induce specific behavioral changes

Biopesticide A naturally occurring substance (or pesticide) from animals, plants, insects or bacteria or even a mineral that can affect the physiology and thereby the behavior of an insect pest species

Biosensor (biological sensor) A device, used for the analysis of a particular substrate; it combines a biological component (enzyme, antibody or nucleic acid) with a transducer that converts the recognition event (or molecular reaction) into a measurable signal

Biotransformation enzyme An enzyme that mediates a specific change of a drug or molecule within a given tissue of a living organism

Bitter taste sensation An acrid biting sensation in the gustatory modality that is associated to activation of bitter taste receptors

Bombykol (*E*,*Z*)-hexadecadien-1-ol, the first ever described sex pheromone, discovered by Butenandt et al. (1959), that is released by the female silkworm moth to attract specifically the male silkmoth

***Bombyx mori* L.** (in French *le ver à soie*) The silkworm of the mulberry tree, symbol of Asia and primary producer of silk and model organism in the study of genetics, neurobiology, olfaction and pheromone

Ca^{2+} channel An ion channel which has selective permeability to calcium (Ca^{2+}) ions

Calcium influx A massive entry or arrival of Ca^{2+} ions inside the cell

Calling The behavior associated to pheromone release; at a precise moment of the night, female moths immobilize on a vertical support such as the stem of a green plant and devaginate a (pheromone) gland located at the abdominal tip; this is accompanied by continuous vigourous wing fanning presumably to help disperse the odor

Calmodulin (calcium-modulated protein) A multifunctional intermediate Ca^{2+}-binding protein that mediates various metabolic processes in insect and other eukaryotic cells

Calyx (from Greek kálux = "husk or pod") A flattened cap of neuropiles in the insect brain where most sophisticated computations occur for signal recognition

cAMP (3′,5′-cyclic adenosine monophosphate) A second messenger important for signal transduction in many organisms, including insects

Capacitance The ability of the neural circuit to collect and store energy in the form of an electrical charge

Carbamate An organic (toxic) compound derived from carbamic acid (NH_2COOH)

Carbon The key ingredient for most life on Earth, the elemental composite of the cell

Carbon dioxide A colorless gas made of a carbon atom attached to two oxygen atoms (CO_2) that occurs naturally in earth's atmosphere and water resources since Precambrian period (about 600 Mya)

Carbon world An unstable ancient world that has influenced evolution and perhaps can be found in the modern time rich in carbon samples, molecules and emissions

Carboxylesterase (carboxyclic-ester hydrolase) An enzyme that utilizes two substrates (carboxylic ester and water) to release two products (alcohol and carboxylate)

Central olfactory pathways A combination of multiple interconnected olfactory structures in the insect brain that processes odor information and triggers specific odor-guided behavior

Chain shortening The process by which a long carbon fatty-acyl (lipid) chain precursor is subjected to selective two-carbon chain reduction to produce a specific sex pheromone

Chemical barrier A fatty acid, a protein, a secretion or another substance that helps defend the body against pathogens

Chemical defence A life history strategy of insects, plants and many other organisms to produce toxic or repellent molecules against predatory attacks; it also includes chemicals that reduce plant (or insect) digestibility to avoid consumption

Chemoreception The sensory modality tuned to volatile and non-volatile chemical stimuli molecules

Chemosensory organ An organ that is able to detect the presence of specific chemicals or relates to the perception of chemical substances – In mammals including human it includes the main olfactory epithelium (MOE) and the vomeronasal organ (VNO); in insects it includes the antennae, legs and proboscis, but not essentially the gut, the fat body, the dermis (immune organ) or the pheromone gland

Chemosensory Protein (CSP) A family of small soluble proteins (four-Cys) ubiquitously expressed throughout the whole insect body, also in arthropods and bacteria, highly abundant in chemosensory organs as well as in other tissues, tuned to fatty acids and xenobiotic chemicals for multiple functions including development, digestion, metabolism, pheromone production and immune defense

Chronobiology The study of the periodic (cyclic) phenomena, of the biological rythms and of the effects of time on living organisms

Cicadella (green leafhopper) A jumping insect pest known to consume sugar on leaves of trees and many various other plant cultures

Circadian clock (*circa diem* = "about a day") An internal clock whose biochemical, genetic and molecular components drive specific changes in the insect behavior depending on rythms with a period close to 24 h

Circadian clock gene A gene that encodes a protein involved in circadian clock oscillation

Circadian rhythm A 24 h cycle in a physiological process within a living organism such as an insect or a plant

Circadian rhythm of pheromone production/emission A 24 h cycle in the regulation of pheromone production and release in mating behaviors of insects (moths)

Cis-7-dodecenyl acetate (Z7-12:Ac) A crucial pheromone chemical for male response to female sexual odors in moths (*Agrotis* noctuids)

Cis-9-tetradecenyl acetate (Z9-14:Ac) A second crucial pheromone chemical for male response to female sexual odors in *Agrotis* noctuid moths

Cis-11-hexadecenyl acetate (Z11-16:Ac) A third crucial pheromone chemical for male response to female sexual blend of odors in *Agrotis* noctuid moths

Cockroach (*blatta* = "insect that shuns the light") A very ancient type of insect (320 Mya) closely associated to food residues and human habitats (since Antiquity); it can adapt to various kinds of external environments such as cold and heat, adopt a social organization, a kin recognition, a group or swarm behavior, a collective decision-making for food choice, and a very peculiar courtship ritual in which the female (*Periplaneta*) eventually climbs on the male's back to devour the abdominal tergal gland, site of production for the sexual pheromone

Cognate ligand A ligand that is strictly required for protein interaction and function

Coleopteran An insect or species that belongs to the order Coleoptera (beetles)

Consensus A motif of conserved amino acid residues in a protein gene family

Contact pheromone (cuticular pheromone) A non-volatile odor or pheromone detected by direct contact with chemoreceptors on the antennae or tarsi of insects and thereby closely related to social insect species such as ants and termites

Courtship ("*faire la cour*") An attempt or a specific behavior of the male to seduce the female in a purpose of mating for reproduction

Cricket (gryllid) A type of nocturnal insect known for the song of males in search for mates and for a sophisticated hearing tympanic system (eardrums) on the front legs

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/Cas) A system or technology for gene/genome editing based on archaeal and bacterial prokaryotic defense mechanisms against foreign viral DNA contamination

Crop protection A field research in agronomy and agricultural sciences for sustained development and high-throughput production of food supply, transgenic plants and leguminous cultures resistant to insects

C-terminal pentapeptide motif The region of PBAN with pheromonotropic activity

Current clamp recording An electrophysiological method for measuring the voltage across a cell membrane at a fixed current across the membrane

Cuticle (exoskeleton) The outermost part (the external armur) of the insect body, also in all arthropod invertebrates, involved in many functions such as defence against toxic chemicals and prevention of water loss

Cycle The time necessary for a sequence of a recurring succession of biological events or phenomena such as those associated to diapause and reproduction to be completed

Cysteine ((R)-2-Amino-3-mercaptopropionic acid) The amino acid residue (Cys) that harbors a sulfur atom and helps build disulfide bridges in specific protein structures

Cytochrome P450 (CYP450) A superfamily of enzymes that use a variety of small and large molecules as substrates in various chemical reactions from the electron transfer chain and exogenous toxic chemical degradation

Cytoplasmic incompatibility A phenomenon caused by bacteria living in the cytoplasm of gamete cells that results in sperm and eggs being unable to lead to viable offspring

Damage The harm, injury, impairment, loss or destruction in biological function or economic value of a sensory cell or an agricultural parcel

Danaus plexippus (monarch butterfly) An iconic pollinator species known on the American continent for winter mass migration

DDT (dichloro-diphenyl-trichloro-ethane) An organochlorine (chlorinated hydrocarbon) insecticide molecule known to be associated with Alzheimer's disease

DEET (N,N-diethyl-meta-toluamide) A renown insect (mosquito and tick) repellent molecule with some known secondary toxic effects on human

Dengue hemorrhagic fever A severe outcome of dengue disease resulting in bleeding, low levels of blood platelets and blood plasma leakage

Dengue shock syndrome A severe outcome of dengue disease, where dangerously low blood pressure occurs

***De novo* pheromone biosynthesis** (*de novo* = "from a new") The particular biochemical pathways in which specific metabolites (pheromone products) are newly biosynthesized typically from acetyl coenzyme A in the (moth) pheromone gland

Desaturase (fatty acid desaturase) An enzyme that removes two hydrogen atoms from a fatty acid, *de novo* producing a specific carbon/carbon double bond pheromone molecule

Detoxification The process of removing exogenous foreign toxic (xenobiotic) substances from an organism, a tissue or a cell

Deutocerebrum (from greek deuteros = "second") A part of the insect brain with numerous glomeruli (ball-like structures) where the axons of antennal receptor neurons end and connect with interneurons and with neurons projecting to higher brain centers; within a glomerulus the receptor neurons of similar odorant specificity converge, there is e.g. one glomerulus for CO2 receptor neurons, pheromone receptor neurons converge in the macroglomerular complex

Development (simple or incomplete metamorphosis) The biological process that all insects must undergo from eggs to the adult stage and reproductive status

Diapause A physiological state dormancy; a delay in development in response to regularly and recurring periods of adverse environmental conditions

Dipteran An insect or species that belongs to the order Diptera (flies and mosquitoes)

Disparlure A specific noctuid sexual pheromone (2-methyl-7,8-epoxyoctadecane) released by female gypsy moths, *Lymantria dispar*

Disulfide bridge A linkage (or bridge) enrolling a disulfide (S-S) bond usually derived by the coupling of two thiol (R-SH) groups within the same protein and/or two different molecular complexes or protein units

Drosophila melanogaster The common fruit fly or vinegar fly, most widely used model organism for biological research in immunology, genetics, life history evolution, trait inheritance, microbial pathogenesis, neurophysiology, olfaction, vision and neurorobotics

Drosophila suzukii The spotted wing *Drosophila*, major fruit, grape, cherry and berry crop pest species worldwide; it is the rare fly that infests fruit and berry during the ripening stage, in contrast to most species of flies that infest only rotting fruit

Duplication (gene or chromosomal duplication) A major mechanism through which new genetic (DNA) material is generated during molecular (genome) evolution through unequal crossing-over (misalignment of chromosomes) and/or retrotransposition event

Dysgeusia The alteration in taste and recognition of gustatory molecules

Eciton hamatum A species of army ant (Dorylinae) known to prey on the larvae of other social insects such as wasps and ants of genera *Dolichoderus* and *Camponotus*

Ecodrug A drug, chemical, agent or reagent with eco-safe property (see ORSA), which needs to be considered for insect control and ecosystem preservation; the required alternative to insecticides and other environmental pollutants

Ecosphere An Earth closed ecological system; The part of the atmosphere in which it is expected to breathe naturally without aid, cure or protection

Electroantennogram (EAG) A recording from insect antennae with both electrodes located within the hemolymph (blood space) but at different regions on the antenna; the voltage changes observed upon odor stimulation reflect mixed responses of many receptor neurons, including temperature effects

Electroantennography An electrophysiological technique for measuring EAG, the average output (sum of responses of many olfactory neurons activated) of an insect antenna exposed to a given odor

Encephalitis An infectious disease in human characterized by a sudden onset inflammation of the brain or the brain tissue

Endectocide Insecticide applied to the host to kill an endo- or exoparasite

Entomopathogen A chemical drug or a bacterial organism that can cause disease specifically in insects

Entrainability The ability of oscillators (or clocks) to be synchronized with an external periodic signal such as seasonal variation and/or day length (photoperiodism)

Enzyme kinetics The study of the chemical reactions and reaction rates that are catalysed (governed) by specific enzymes

Euarthropoda The phylum of "true" arthropods (arachnids, crustaceans, insects and myriapods); their cuticle is periodically shed to allow for continued growth

Exon A part of a gene that will encode a part of the protein (block or motif) after introns have been removed by RNA splicing

Fairyfly (fairywasp) A family of almost invisible beautiful very tiny insects with a feathery appearance, the most primitive family within Chalcidoidea (100 Mya), which has a very short lifespan at the adult stage; females have the antennae tipped with club-like segments (clava), while the male antennae are filiform and look like a long soft cotton fiber thread

Fatty acyl reduction The chemical process involving the gain of electrons in a fatty acid to yield a fatty alcohol (via a fatty aldehyde intermediate)

Food choice An impact that food, plant or prey selection has on the environment, health and life of many organisms

Food trail pheromone An odor chemical that builds a narrow and precise route for the (insect) organism to reach specific food sources

Formica rufa The red wood (or horse) ant that sprays formic acid from their abdomen

Free-running period A period or rythm that is not adjusted to 24 h cycle nor to any other artificial photoperiodic cycle

Glutathione-S-Transferase (GST) (ligandins) A family of metabolic enzymes that catalyze the conjugation of the reduced form of glutathione (GSH) to foreign xenobiotic substances, participating thereby to cell or tissue detoxification

GABA (gamma-aminobutyric acid) A neurotransmitter that acts at inhibitory synapses by binding specific receptors in the membrane of both pre- and postsynaptic neuronal processes; it regulates brain and nerve cell (neuron) activity by decreasing the number of neurons firing in the insect (and human) brain

Genetic code Building blocks of life (Watson and Crick 1953); Genetic information in DNA conveyed solely by the linear sequences of four (nucleotide) bases (A, T, G and C) in a triplet codon alphabet that is used by living cells to translate gene/RNA into protein (most of all amino acids in the protein are specified by more than one codon or nucleotide base triplet in the DNA = degeneracy of the genetic code)

Genome A complete set of DNA (genes, exons and introns) that contains all the information necessary to build an organism and lead its activity through expression of a complete and specific repertoire of proteins

Glomerulus (*glomus* = "ball of yam") A globular structure or neural network of entwined vessels, fibers and nerve cells (neurons)

Glutamate An excitatory neurotransmitter in the (insect) brain essential for normal brain function, learning and memory

Glycine (aminoacetic acid) The simplest possible amino acid residue (Gly) that has a minimal side chain (one single hydrogen atom) and therefore can fit into any hydrophilic (attracted to water) or hydrophobic (not attracted, even repulsed by water) medium; it has a repeated role in the modulation of alpha-helical motifs in many various proteins

G-Protein Coupled Receptor (GPCR) A protein located in the cell membrane compartment (seven transmembrane domains) that binds extracellular substances and transmits specific signals though an intracellular relay molecule called G-protein (guanine nucleotide-binding protein)

G protein-coupled receptor kinase 2 (GPCRK2) A family of protein enzymes that regulate the activity of GPCRs by phosphorylation/dephosphorylation process

Guillain-Barre syndrome (GBS) A rare disorder caused by the immune system damaging the peripheral nervous system (= nerves outside the brain and spinal cord)

Gustation One of the five senses that belongs to the gustatory (taste) system

Haplotype A group of genes that are inherited together from a single parent

Heliothis virescens (tobacco budworm) A species of noctuid moths whose larvae are addicted to gluttony on cotton, pea, soybean and tobacco with extremely high resistance to a large panoply of insecticides

Hemocyte A cell from the hemolymph that plays a role in the immune system of insects (analogous to human phagocyte)

Hemolymph A transport fluid from the circulatory system that fills in the body cavity and all tissues in insects as well as in other arthropods (rather analogous to human lymph, not to human blood); it does not help carrying oxygen, it helps fighting infections and removal of waste toxic products

Histamine A biogenic amine inhibitory neurotransmitter in the insect brain

Honey bee (*Apis mellifera*) the most beneficial insect for human; building most intimate interactions with flowers, it provides human with honey, beeswax and crop pollination

Host plant odor A specific odor profile released by a plant most suitable for the moths or butterflies that need to lay eggs on it

Host preference The choice of an insect to find most suitable individual, organism, species, nest or plant for blood meal, food source, egg-laying and/or reproduction

Host selection The use of both olfactory and visual cues in (plant) host location

Hyalophora cecropia (cecropia moth) A giant silk moth with beautiful feathery antennae used to detect pheromonal odors from miles away. Also known for the discovery and extraction of juvenile hormone (1956) and as a symbol of North-American natural fauna

Hydrophobic semio-chemical A chemical signal used a mean of communication between organisms that can dissolve in the air, but not in the water

Hymenopteran An insect or species that belongs to the order Hymenoptera (ants, bees, sawflies and wasps)

IMD (immune deficiency) A key component of the immune response to infection specifically in the insect gut

Immunity The ability of an organism (including insects) to resist an infectious agent, a pathogen, a toxin or toxic xenobiotic substance by the action of the immune system

Inhibition of receptor neurons Nerve impulse firing possibly inhibited (i) by poisons affecting the nerve impulse generation (e.g. permethrin), (ii) by antagonistic ligands blocking odorant receptor molecules (e.g. presumably decanoyl-thio-1,1,1-trifluoropropanone selectively inhibiting pheromone-sensitive neurons of moth species), and (iii) by odorants that produce receptor potentials of opposite polarity thereby decreasing the spontaneous nerve impulse firing (e.g. linalool that inhibits some olfactory receptor neurons but excites others)

Inositol 1,4,5-triphosphate (IP3) (combined with diacylglycerol or DAG) A secondary intracellular messenger molecule used in sensory signal transduction and

lipid signalling that is known to diffuse through the cell to release intracellular calcium stocks

Insect (*insectum* = "with a divided body": head, thorax, abdomen) The largest group within Arthropods (a profusion of species); The most diverse kind of arthropod, characterized by a pair of antennae erected on the head, six legs and one or two pairs of wings at the adult stage- A panoply of developmental and reproductive variations- A set of sophisticated appendages or glands to make sounds or odors – A set of remarkable very sensitive and specialized organs of sensory perception- An example of parasitism or essential beneficial role- Their appearance and survival coincide with first Earth's terrestrial ecosystems (500 Mya)

Insect antennae Paired head appendages carrying numerous sense organs (sensilla) for detecting stimuli of various modalities: odorants, CO_2, taste compounds, mechanical stimuli (e.g. touch, vibration, sound), temperature

Insect behavior A very wide range of innate activities from pheromone communication to reproduction and migration, also including a whole panoply of diverse responses to environmental (toxic chemical) changes

Insect growth regulator A chemical substance that inhibits the life cycle of an insect

Insect pest An insect species that causes specific damages on crops or food supplies or poses a real threat to human health

Insertion mutation A type of base (or amino acid) mutation characterized by the insertion of one or few nucleotide base pairs to a DNA or RNA strand and/or the insertion of one or few amino acid residues (Glycine) to a protein motif or structure

Intron The silent (non-expressed) part of a gene, laying between two exons; it helps assemble exons but is removed from RNA after maturation before protein synthesis

Inversion mutation A type of base (or amino acid) mutation characterized by the removal of a length of DNA or a pair of amino acids which is then reinserted in the opposite direction in a protein motif or structure

Iodobenzene An organic compound with a benzene ring and one iodine atom

Ion channel A protein of the cell membrane serving as a gate for ion currents across the membrane; it may be opened upon specific (odor or neurotransmitter) ligand binding

Ionotropic receptor (ligand-gated ion channel) A family of ion-channel proteins located in the cell membrane which allow ions (Na^+, K^+, Ca^{2+} and/or Cl^- to enter the nerve cell in response to the selective binding of a chemical messenger neurotransmittor (or ligand)

Ipsdienol The aggregation pheromone ((4S)-2-methyl-6-methylideneocta-2,7-dien-4-ol) of bark beetles

Juvenile hormone (JH) A (main) hormone in insects, secreted by two tiny translucent endocrine glands near the brain (*corpora allata*), which play a crucial role in controlling most of the key processes in the insect physiology from development and molt to growth and reproduction through chemical communication, migration and oviposition

Juvenile hormone binding protein (JHBP) A protein that interacts with or helps the transport of JH in the hemolymph or in different compartments of the target cell to control specific gene expression

Kenyon cell An intrinsic nerve cell (or neuron) from the mushroom body of insects

Labial palp pit organ glomerulus The part of the insect brain tuned to CO_2 detection

Lamellocyte A large flat cell of the insect immune system that is known to function as a plasmatocyte (hemocyte)

Lateral horn (*lateral protocerebrum*) One of the two areas in the insect brain (the other area is the mushroom body) where projection neurons of the antennal lobe send their axons and specific odor information

Lepidopteran An insect or species that belongs to the order Lepidoptera (butterflies and moths)

Leucophaea maderae (Madeira cockroah) The first organism where an endogenous circadian clock was identified

Ligand-induced internalization An uptake of a material into a different compartment

Ligand-induced internalization of Ca^{++} into a receptor neuron A mechanism (desentization process) controlling odorant receptor signaling to ensure the appropriate cellular responses to a specific odor molecule

Linked gas chromatography-electrophysiology A technology that combines separation of pheromone volatile chemicals vaporized without decomposition (gas chromatography) and recordings from single olfactory neurons (electrophysiology) to screen for biological natural active novel compounds

Lipids A group of (oil, fat, wax and other ester) organic compounds strictly insoluble in water (highly hydrophobic); it is (with carbohydrates and proteins) the primary structural component of living cells

Local neuron (interneuron) A broad class of nerve cells that enable communication between sensory neurons and the central nervous system in the insect brain

Locomotor activity rhythm (*locō* = "from a place") A strong regular repeated pattern of movement from one place to another, largely under the control of a persistent endogenous timing mechanism of circadian frequency

Locust A solitary or gregarious insect (grasshopper) that can migrate in gigantic swarms and cause immense damages on cultures, vegetations and crops

Locusta migratoria The migratory locust that can change characteristics or traits (phenotype; from solitary to gregarious) in response to population density and build swarms of 40–80 millions individuals

Log_{10}-unit of stimulus intensity A step of factor ten in stimulus strength

Lymantria dispar (gypsy moth) The most destructive pest (Lymantriidae) of hardwood trees in US and North-America

Lymph emulsion A water-in-oil emulsion; a suspension of lipid droplets of oil in a water environment with which the oil will not mix

Maculopapular rash A type of rash characterized by a flat, red area on the skin that is covered with small confluent bumps

Mamestra brassicae (cabbage moth) An invasive noctuid species of moth known to feed (at the caterpillar stage) on many various fruits, vegetables and crops (cabbage, broccoli, Brussels sprouts, tobacco, tomato, sunflower, etc)

Management The process of dealing with or controlling insect pests

Manduca sexta (hawk moth; in French *le sphinx*) A species of moth (Sphingidae) that feeds on flowering plants (Solanaceae or nightshades) from agricultural crops, medicinals, spices, weeds and ornamentals, and a common model organism in odor neurobiology

Mating The action of pairing for intersexual interaction or reproduction

Microcephaly A medical condition present at birth or later during the first few years of life in which the brain does not develop properly resulting in an abnormally small head

Microfilaria An early stage in the life stage of parasitic nematodes (worms) that can be taken up from an individual (host) by blood-feeding insects and develop to infective larvae transmitted to a new host prone to cause epidemic diseases

Migration Seasonal flights or movements of insect species such as beetles, butterflies, dragonflies, locusts and moths (most damaging) in response to environmental changes

Molecular receptive range The agonist (excitatory) and antagonist (inhibitory) characteristics of an odorant receptor

Mosquito A long-legged buzzing dipteran fly with aquatic larvae and female that feeds on human blood transmitting a series of serious epidemiological infectious diseases (Chikungunya, Dengue, Malaria, Zika, etc)

Moth A crepuscular or nocturnal insect species with gluttonous herbivorous (phytophagous) larvae, females with pheromone gland at the abdominal tip and males with prominent hairlike or feathery antennae which flies at night to find the females that emit the odor over kilometers distance

Multiglomerular structure A (brain) structure that affects, contributes or pertains to multiple glomeruli

Musca domestica (house fly) The most common species found on cattle farms, a nuisance that can transport vector-mediated diseases; it is also a key element in ecological chain for breaking down and recycling organic matters

Mushroom body (*corpora pedunculata*) A pair of nervous structures in the insect brain known to play a key role in olfactory learning and odor memorization

Mutation A change, not necessarily an alteration, in the DNA, RNA or protein sequence that helps produce a new gene, RNA or protein isoform, prelude to new function in a given gene protein family in responses to specific external environmental changes

Myalgia A pain in one or more muscles

Mymar pulchellum A genus of fairyflies in Euathropoda Insecta Hymenoptera Mymaridae (only ten species described)

Mythimna separata The rice-ear cutting caterpillar; the major pest of maize in Asia

Negative staining The staining of the background used in transmission electron microscopy in order to increase contrast to the specimen

Nerve impulse (of sensory neuron) An action potential elicited (or suppressed) by the receptor potential reaching the impulse generator zone; this zone is thought to be located in the soma (cell body with nucleus) of the neuron, nerve impulse may also be spontaneously generated

Neuropile An area in the insect brain or any nervous system composed mainly of nerve fibers (only a few nerve cell bodies) that forms a synaptically very dense region

Niemann-Pick type C2 protein (NPC2) A small soluble β-stranded protein important for cholesterol, fatty acid and sphingolipid transport in the lysosome of animal cells and the sensory lymph of ant workers

Noxious compound detection The sensory perception of chemicals that are harmful, eventually destructive and difficult to control or eliminate (toxicants)

Noxious compound protection A mechanism in the insect defense system that allow them to cope with the toxic secondary compounds from the plant for specialization, selection and specific adapation to a potentially new habitat (host)

Nuptial gift A piece of food, twig of wood, tuft of grass or very precious bowl of silk that is given by the insect male to the female prior to mating

Octopamine The insect noradrenaline; it regulates aggression, behavioral development, reproduction, sleep, flight and odor memorization in various insect species, modulating specific neural signals in olfactory learning and memory as well as circadian rythms of sleep and activity for instance in honey bees, fruit flies and crepuscular moths

Odonatan An insect or species that belongs to the order Odonata (damselflies, dragonflies and Libellulidae)

Odor A scent, a stench, a bad or neutral smell that is caused by one or more in a bouquet airborne chemical volatiles all perceived by the sense of olfaction (i.e. the human nose or the insect antennae); it eventually refers to fragrance (a flower aroma, a perfume, a good positive enjoyable smell) for the positive aspect of life

Odor discrimination The perceptual ability (of the brain) to detect and describe differences between odors or perfume scents

Odor perception The brain's interpretation of the activation responses of many peripheral sensory neurons from the human nose or insect antennae which are differentially sensitive to a wide variety of molecules or chemical odorants

Odorant-binding protein (OBP) A small soluble α-helical protein that binds to odor molecule (odorant) at the periphery of olfactory receptors in the insect antennae

Odorant clearance The process of removing (eliminating, cleaning out, washing out etc) any residual odorant molecule from the human nose or the insect antennae

Odorant-Degrading Enzyme (ODE) An enzyme that mediates the metabolism of volatile signal molecules crucial to sustained sensitivity and specificity in the insect olfactory system

Odorant inactivation (odorant deactivation) A chemical alteration of odorant molecules by specific enzymes (ODEs) that stop them interacting with receptor molecules

Odorant Reception Suppressing Agent (ORSA) An airborne volatile or non-volatile synthetic odor pheromone chemical structural analog with a subtle modification in the native molecular stretch for the ability to block specifically the functional binding sites of target olfactory proteins and/or to counteract with specific odor receptor activation

Odorant receptor (OR) (olfactory receptor) A seven-(pass)-transmembrane domain protein expressed in the cell membrane of olfactory (receptor) neurons that need to be activated by specific odor molecules before the sense of smell

Olfaction The sense of smell; the primary sense tuned to odor detection and recognition; One of the most ancient and primordial modality to sense the environment

Olfactory co-receptor (ORCO) A co-expressed and co-localized olfactory receptor protein that complexes with odorant receptor to form an odorant-sensing unit

Olfactory receptor neuron (ORN) (olfactory sensory neuron, OSN) The cell that transduces chemical odor signals into electric neural messages that are sent out to the brain for odor sensing (ten million in human, thousands to ten thousands in insects)

Ophthalmotropic (from greek ophthalmos = "eye" and tropic = "turned towards") An insect species (moths or flies) that have developed feeding habits and mouth parts typically tuned to animal eye secretion

Optic lobe A structure or pair of structures (left and right) found in the microbrain of insects that integrate sensory information from the eyes and certain auditory stimuli

Organophosphate The common name for phosphate esters or esters of phosphoric acid; it includes DNA, RNA and ATP but also most common insecticide phosphorous chemical

Orthopteran An insect or species that belongs to the order Orthoptera (crickets, grasshoppers, katydids and locusts)

Ostrinia nubilalis (in French *la pyrale du maïs*) The European corn borer (*E* and Z strains); A grass moth (Crambidae) pest of grain, known for hairbrushes or hairpencils (aphrodisiac organs) in the middle and lower abdomen that the male opens out like a fan during courtship to facilitate its acceptance by the female

Oviposition The act or behavior related to lay eggs in insects

Palindrome A DNA or protein sequence that is spelled the same way forwards or backwards

Parasitoid An insect (usually a wasp) whose larvae feed and develop within or on the body of another insect species (usually a moth caterpillar): an example of endoparasitism (when the parasite lives inside the host organism)

Patch clamp recording A voltage or current clamp recording with the mouth of the recording electrode tightly sealed (GOhm seal) to a small patch (piece) of the neuron plasma membrane containing one or a few ion channels

Pathogen An agent such as a virus or a bacterial microorganism that can cause infectious disease

Pattern recognition receptor (PRR) A protein expressed by the (insect) innate immune system that plays a role as a host-sensor; it detects molecules specific to pathogens

PBAN agonist A peptide molecule that can bind to and activates a PBAN receptor to induce (or stimulate) a PBAN response

PBAN/pyrokinin family (FxPRL amides) A large family of neuropeptides (PBAN, diapause hormone, melanization and reddish coloration hormone-MRCH, myotropin, etc) that bear the same amidated C-terminal tail (FxPRL) and regulate multiple various physiological functions in insects (Lepidoptera), i.e. development, cuticular coloration, flight, mating, muscle contraction, pheromone production and wing tanning

PBAN receptor (PBANR) A G-protein coupled receptor with seven-(pass)-transmembrane domains which triggers a specific signal transduction in the female moth pheromone gland leading to pheromone production in response of PBAN activation

Pedunculus (Peduncule) A stemlike structure that collects nerve fibres and thereby connects different regions from the central nervous system of the insect brain

Peptidomimetic A subtly modified peptide chain that mimics the effect of the natural peptide or a system similar to peptides (poly-N-substituted glycines or peptoids and amyloid β, Aβ or Abeta peptides)

Period A gene that is expressed in a circadian pattern to associate specific behaviors with circadian rythms, the primary circadian pacemaker in the insect brain

Peripheral clock A functionaly autonomous local oscillator in circadian timing active not in the brain but in many peripheral organs or tissues such as the gut and antennae of the insect influenced by light, temperature, hormonal regulation and/or fasting-feeding cycle

Periplaneta americana (American cockroach) The largest pest species of common cockroach with ability of limb regeneration at the nymphal stage, a cosmopolitan plague that can live more than a year, reproduces over six hundred days and leads to more than ~150 progenies/a year

Perireceptor event The interaction between two or more molecular elements (ligand, transport protein, scavenger protein, enzymes) at the periphery of the receptor protein with central or pivotal function (i.e. odor receptor in the olfactory system)

Perireceptor event in insect olfaction The extracellular processing of the odor molecule before and after its interaction with the receptor protein, such as binding to soluble odorant binding protein, transport and degradation by odorant-degrading enzyme

Peritrophic matrix A semi-permeable envelope of chitin microfibriles that surrounds food metabolites in the insect midgut essential for digestion and infection by pathogens

Permian The geologic period of time and system which spans about 50 million years from the Carboniferous period (about 300 Mya) to the beginning of Triassic (about 250 Mya); it corresponds to the largest mass extinction of life recorded in the history of Earth (also called the Great Dying: 96% of species died out), the end of Paleozoic era

Pherokine A molecule related to both pheromone and immunological systems

Pheromone (from Greek phérein = "carry", and hormáo = "to set in rapid motion, stir up", "hormone") A secreted or excreted odor molecule, an odorant factor or chemical signal that triggers a specific behavioral response in individuals of the same species

Pheromone Biosynthesis Activating Neuropeptide (PBAN) A neuropetide (33 amino acids) with functional C-terminal FxPRL-NH_2 tail produced in the insect head (suboesophageal ganglion) secreted via *corpora cardiaca* (neurohemal organs of insects) and released into the hemolymph (and/or the ventral nerve cord) for the induction and stimulation of *de novo* pheromone biosynthesis in the lepidopteran female moth pheromone gland at some crucial time of the night

Pheromone blend A few or multiple pheromonal odors aimed at combining different molecules into a species-specific uniform whole odorant signal

Pheromone degrading enzyme (PDE) An enzyme that specifically mediates pheromone degradation (catabolism) and/or the conversion of pheromone molecules into inactive (or less active) forms

Pheromone gland A primary source and reservoir for sequestrating *de novo* biosynthesized chemical compounds with pheromone function (e.g. the sex pheromone gland of female moths); it is usually covered by pines on the gland surface to facilitate pheromone emission and/or odor release

Pheromonogenesis The genesis of *de novo* (sex) pheromone chemicals via multiple key biosynthetic enzymes from the uptake of fatty acid, lipid or thioester precursor molecules to the final product of specific pheromone biosynthetic pathway

Pheromonostasis A mechanism or a peptide molecule mediating arrest or suppression of pheromone production in the sex pheromone gland; it naturally occurs in female moths after mating thanks to a number of humoral (male factors, sex peptides) and neural cues, it can also be induced by a family of biosynthetic sex peptide analogs inhibitors of sex pheromone production in selected insect pest species

Phospholipid A large biological polymer of the lipid family with hydrophobic "legs" (fatty acid) and hydrophilic "head" (phosphate) that plays a crucial role in the formation of cell membranes and all membranes surrounding organelles (= cell organs, differentiated structures within a cell that performs a specific function, e.g. mitochondria from the insect cell)

Phosphorylation The reversible process of attaching a phosphate group to a molecule (mainly on Serine, Threonine or Tyrosine amino acid residue) to help lead a protein to trigger a specific physiological mechanism (opposite: dephosphorylation); it is certainly one of the most important post-translational modification in various protein structures, including enzymes and receptors

Photoperiod The lenght of day or night in a cycle of time (24 h)

Photoperiodic clock An endogenous (internal) clock or timekeeping network that allows insects as well as many various organisms to align a specific physiological system with a changing external environment in order to perform most adapted biologically relevant important behavior

Photoperiodic response A functional physiological and/or behavioral change in response to a change in the length of day and night

Photoperiodism The physiological reaction of insects (and plants) to a photoperiod

Physical barrier An environmental, induced or natural condition that interferes in communication or interaction between two cells, individuals, organisms or species

Physiology The discipline of biology concerned with the functioning of living organisms

Pit organ A temperature- infrared- CO_2- and odor-sensitive organ on the antennae, or antenniform legs of insects (beetles, hymenoptera, moths), the small Haller's organ on the forelegs of ticks and varroa used to detect heat and pheromone chemical odors released by host (honey bee); it is formed by a ring-shaped cuticular ridge surrounding a pit (a hollow or indentation in the leg surface) containing five or six raised pore openings within each two to five sensilla are exposed

Plant-herbivore insect interaction A range of adaptations evolved by plants and insects for co-evolution: the responses of the plant to herbivore insect attack, the responses of the insect to plant defense, host-plant resistance, insect resistance, survival dynamics

Plant semiochemical (from Greek semeion = "signal") A chemical substance released by plants to defend themselves against herbivore insect attack by reppeling the assaillant and/or by attracting natural ennemies (predators) of the herbivore (tritrophic interactions)

Plasmodium falciparum A unicellular protozoan parasite transmitted by *Anopheles* mosquitoes that is the main cause of malaria (anemia) disease in humans

Poison avoidance The act of avoiding (keeping away) from toxic chemical element possibly ingested (by insects) though food and nutrients

Poisson statistics Statistics of random events as e.g. arrival of single stimulus molecules on olfactory sensilla at a weak stimulus concentration

Proboscis The insect tongue (the sucking organ of a bee, a butterfly, a fruitfly or an hawk moth); an appendage, elongation or extension at the front of the insect mouth whose vital function remains elusive in most adult moths as most adult moths do not feed and do not suck nectar: proboscis should be absent when superfluous

Projection neuron An afferent (arriving to the brain) or efferent (exiting the brain) axonal projection fiber nervous cell uniting the insect brain with lower parts, peripheral nervous system, suboesophageal ganglion (SOG) and other ganglia of the ventral nerve cord that innervates (for instance) the pheromone gland in moths

Proline (pyrrolidine-2-carboylic acid) The only amino (imino) acid residue (Pro) with a pyrrolidine (or tetrahydropyrole) and amine function for side chain, which confers an exceptional conformational rigidity in protein structure; it is usually found at the beginning of alpha-helices and in the edge strands of beta-sheets: polyproline motifs are essential for protein phosphorylation, protein assembly and signalling

Protein The core of life in cells (with lipids and other molecules); a short or very elongated soluble or trans-membrane macromolecules consisting of one or multiple chains of amino acid residues (such as Cysteine, Glycine and Proline) that

combines to build the (primary) structure dictacted by the nucleotide sequence of the corresponding gene on the basis of the genetic code (Watson and Crick); specific amino acid motifs can adopt different types of (secondary) structures (alpha-helix, beta-sheet and beta-turn) and foldings (tertiary structure) to underlie specific cell functions in adhesion, cycle, development, division, growth, shape, catabolism, metabolism, transport, regulation, signalling and immunological responses; to fullfill these tasks in multiple systems, proteins are often subjected to post-translational modifications (see phosphorylation) and it is said that the protein can even be subjected to specific (Cys, Gly or Pro) insertion mutation or inversion to acquire multi-function

Protein structure model (homology-modelling) An inference of protein's tertiary (3D) structure (prediction of alpha-helices and variations) from its amino acid sequence based on the known 3D crystal structure of a homologous protein used as reference or template

Protein variant (protein isoform) A representation of changes (mutations) in the amino acid sequence encoded by a specific DNA sequence (gene) in the genome; A new protein sequence in the repertoire of highly similar proteins that originate from the same gene but differ by one or a few amino acid replacements, the simplest variant (isoform or mutant) being the protein in which only one amino acid was subtly replaced by another to induce a new protein function

Protocerebrum The region of the insect brain innervating the compound eyes; it includes important higher centers like the mushroom bodies and the central body

Protozoan A rather informal term to refer to unicellular eukaryotic organism (or protist); a main class of parasites that cause infectious disease (Malaria) in human

Pyrethroid An organic insecticide compound similar to the natural pyrethrin molecule from pyrethrums flowers (*Chrysanthemum cinerariaefolium*)

Receptor potential A change of electrical voltage indicating the excitation of a sensory neuron, the stimulus-induced change of neuronal membrane conductance; it may be recorded extracellularly using capillary electrodes, with the "indifferent" reference electrode in contact to the hemolymph or blood space, and the "recording" electrode positioned near to the apical portion of a sensory neuron. The polarity of the receptor potential is negative or positive if the neuronal membrane conductance is increased or decreased upon stimulation

Receptor potential/current, elementary (ERP/ERC) An elementary small transient voltage/current wave ("bump" or group of "bumps") elicited by a single odorant molecule or (infrequently) spontaneously

Repellent An odorant chemical molecule that can elicit an aversive or repulsive behaviour specifically in some insect pests or predators

Reproduction The biological process by which a new individual organism (descendant or offspring) is produced from a « mother » and a « father » parent; one of the most important concept in biology in which an organism is born and tends to make a copy or a likeness of itself to sustain and give a chance for a species, a genus, a family or an order to survive and/or have a continued existence during the process of evolution

Retrotransposon (transposon via RNA intermediate) A genetic element that can copy and paste itself at many different locations in a genome eventually inducing mutations by inserting near or within a particular gene sequence

Rhizophere The region of soil where interactions between plant roots and associated bacterial microorganisms take place

Rickettsia A genus of bacteria of the tribe Rickettsiae; A small, nonmotile, non-spore forming, highly pleomorphic (occurring in many various distinct forms) rod-shaped to coccoid bacterial organism that lives in the body of lice or ticks and is responsible for Mediterranean spotted fever in humans

Riptortus pedestris An alydid hemipteran insect species (bean bug) extremely polyphagous; one of the major pests on leguminous crops (soybean), whose diapause is tightly regulated by circadian cycle and endogenous clock genes

RNA (ribonucleic acid) A polymeric single-stranded molecule that conveys the information from DNA to protein and therefore represents one essential core for gene expression and cell function; the origin of life: the components (chains of nucleotides, ribose and phosphate) built on crust in space and assembled on Earth

RNA-DNA difference (RDD) (mismatch or mutation) A site of base replacement or switch between DNA and RNA sequences during transcription (= copy of DNA to RNA) or following specific RNA editing by ADAR enzymes

RNA editing The guided post-transcriptional (= after copy of DNA to RNA) subtle modification of RNA sequence from the genomic DNA sequence that can lead to high number of protein variants and thereby multifunction from a single gene

RNA interference (RNAi) A mediated knockdown process in which specific RNA molecules inhibit the expression or translation of a specific gene resulting in the absence of a target protein in a given cell, tissue or organism

Scavenger An insect (fly or wasp) or a protein that feeds on or interacts with the residual matter, keeping a dust-free environment (or fluid) by specific nature recycling processes

Schistocerca americana (American bird grasshopper) The main pest for (palm) trees and lemon crops in Florida, also know for a specific family of fatty acids (caeliferins) from the grasshopper regurgitant that induces the plant to release allelochemicals

Schistocerca gregaria (gregarious desert locust) One of the most dangerous and threatening insect species for humans; it can build swarm of 50–100 billion individuals and can eat up one/tenth of human agricultural production and food supply in three main parts of the world (Africa, Middle-East and Asia)

Second messenger A molecule inside the cell that transmits a specific signal from a transmembrane receptor to an intracellular target (the first messenger being the hormone or the odor chemical that conveys the signal to the cell)

Selectivity The quality of the insect olfactory system of discriminating, selecting and carefully choosing an odor as the most suitable

Seminal fluid (semen) A fluid that is produced by the male reproductory tract secretory tissues (accessory glands, seminal vesicles, ejeculatory duct and testis) and that contains sperm cells (= spermatozoa) and proteins that are transferred

to females with sperm during mating, resulting in specific changes in female behavior and physiology (pheromone inhibition, rejection of male, facilitation of feeding, ovulation and ovogenesis/egg production)

Sensillum (plural: sensilla) A small epithelial sensory unit including a cuticular structure (hair, plate) supplied with (often three) auxiliary cells, and innervated by one or several receptor neurons; in hairlike sensilla (hair length 10–500 μm) the apical neuronal processes (dendrites) may extend throughout the hair shaft, the axons of the receptor neurons conduct the nerve impulses to the central nervous system in the insect brain

Sensillum lymph The aqueous fluid that baths the dendrites of olfactory neurons with pheromone solubilization/emulsification by binding to proteins, pheromone transport and degradation (see perireceptor events)

Sensillum type, gustatory and mechanoreceptive S. chaeticum (bristle, innervated by several taste neurons and often one mechanoreceptor neuron ending at the sensillum base)

Sensillum types, olfactory S. trichodeum (very long hair), s. basiconicum (short hair), s. coeloconicum (very short hair, sitting in a pit), s. placodeum (pore plate, in bees and beetles), *s. ampullaceum* (deeply hidden hair, found in ants for CO_2 detection)

Sensory adaptation A reduction in the responsiveness due to preceding stimulation, observed in responses of sensory receptor neurons and in behavioral responses

Sensory transduction The sum of processes in which a signal chemical (odorant, tastant) induces a receptor potential and impulse firing of a receptor neuron; this may happen via direct gating of ion channels or include a cascade (a series of) molecular events such as protein phosphorylation, second messenger formation, and release of intracellular Ca^{2+}

Serotonin (5-hydroxytryptamine or *5HT*) A monoamine neurotransmitter that acts also as a systemic hormone in insects where it regulates circadian rythms, gut motility, tissue secretion, development, growth, locomotion, flight, learning and memory

Serotype A group of intimately related microorganisms distinguished by a common set of antigens or the set of antigens characteristic of this group

Sex pheromone A long-range highly volatile natural odorant pheromone chemical usually released by the female from a peculiar organ such as the sex pheromone gland of female moths to attract a conspecific male on a precise location, cocoon or plant site prelude to mating and reproduction

Sex pheromone gland A layer of glandular epithelial cells sandwiched between ovipositor and sclerotized cuticle at the tip of the female abdomen; A very active site for lipid and pheromone droplets, specifically devaginated (in nocturnal species of moths) during calling behavior at crucial moment of the night for release of sexual odor volatiles

Species similarity and difference A fundamental resemblance or common point, an homology (a shared ancestry) and/or an analogy (an apparent resemblance of

structures that clearly have different origins but similar function) and dissimilarity (or dissemblance) between different species

Specificity, neuronal The pattern of stimulatory chemicals producing excitatory and inhibitory responses of a receptor neuron; pheromone receptor neurons may have an extremely high specificity in responding >100-fold less sensitive if the pheromone structure is minimally changed, other neurons may respond to a number of chemicals in various proportions

Sphingolipid A lipid with sphingosine (a molecular structure shape as enigmatic as a Sphynx) that accumulates in tissues as diverse as the liver and the brain to regulate diverse cell functions in response to cellular stress (mainly oxidative stress)

Sphinx ligustri (privet hawk moth) The sphinx of the Paleartic zone (Europe and Asia)

Spodoptera frugiperda (*frugiperda* = "lost fruit") The fall armyworm, a severe case study of cannibalism and herbivory in noctuid moths

Spodoptera littoralis (African or Egyptian cotton leafworm) The Mediterranean brocade labeled as quarantine pest (40 different plants and at least 87 different plant species) that feed on young leaves, young shoots, stem, pod, bud and fruit throughout the whole world

Stem cell An adult or embryonic cell that can differentiate into another type of cell and function to produce even more of these new cells and functions

Streptomyces The largest genus of *Actinobacteria* (about 500 Mya); the most adapted organism to the utilization of plant and soil residuum in all various environments

Structure activity relationship The relationship between a chemical (or drug ligand) and/or a 3D structure of a protein molecule and their biological activity

Suboesophageal ganglion (SOG) A part of the ventral nerve cord below the oesophagus inside the head in insects (and arthropods) connected to the brain and to the first thoracic ganglion that controls the mouthparts and salivary glands but also produces neuropeptides (e.g. PBAN) that will stimulate the pheromone gland at the abdominal tip

Sugar taste inhibition The loss of sweet taste perception as a result of the alteration in the activation of sweet taste receptors and/or a neurobiological disturbance in the insect brain or ventral nerve cord

Surface tension The attractive force exerted upon the surface molecules of a liquid by the molecules beneath; it tends to draw the surface molecules into the distinct mass of the liquid and makes the liquid such as water assume a shape with the least surface area (e.g. water or lymphatic surface in contact with air)

Surfactant A solute or substance which tends to reduce the surface tension of a liquid in which it is dissolved

Swarming A collective behavior displayed by insects of the same species (locusts, butterflies, moths, beetles, flies, mosquitoes, aphids, whiteflies, wasps, termites, flying ants and most other winged insects) to aggregate together, move in large numbers and migrate towards specific geographical locations to reproduce or continue development

Synapse (from Greek synapsis = "conjuction") A structure or intercellular space where a neuron (or nerve cell) connects another neuron or a target cell and propagates a specific chemo-electrical signal

Taste (gustation) The primary sense used by human, animals and insects to distinguish one potential food source from another

Taste sensillum A bristle-like sensillum (chaeticum) on the insect maxillary palp (mouthpart) or insect antenna responsible for sweet sugar detection

Temperature compensation A phenomenon in which the output of the endogenous clock system remains nearly constant with fluctuations in external temperature

TEP protein (thioester-containing protein) An antimicrobial protein from the insect immune system that uses a specific thioester motif to damage the cell membrane at the surface of the invading infectious pathogen

Termite Eusocial insect that evolved from an ancestor of cockroaches (about 300 Mya) and entirely tuned to digestion of cellulose that the wood is made of

Tip recording The recording capillary electrode is slipped over the tip of a hair-like sensillum in order to record receptor potentials and nerve impulses from the sensillar neurons; in olfactory sensilla the hair tip may be opened for improving the electrical contact to the neuronal dendrites inside the hair shaft, taste sensilla (sensilla chaetica) have a terminal opening that receives tastants and also allows electrical contact to gustatory neurons and to a mechanoreceptive neuron

Toll receptor An immune receptor in the membrane of sentinel cells (macrophages) from the insect adaptative immune system that can recognize molecules that are broadly shared by pathogenic microbes (sense internal danger signals) and trigger many various responses of the insect defence system, including antimicrobial peptides, proinflammatory cytokines and chemokines

Transcript (mRNA) A single-stranded mRNA product synthesized by transcription of a genomic DNA sequence, eventually subjected to editing and processed for translation (protein synthesis); multiple transcripts or mRNA sequences do no mean necessarily multiple genes, a gene can lead to multiple transcripts and therefore to multiple proteins

Transcription The process in which the genetic information from DNA is transcribed into RNA by a specific enzyme called RNA polymerase

Transepithelial recording A tip recording implemented if the indifferent electrode is located basally from the epithelium; in cases of high electrical resistance across the epithelium (e.g. 200 MOhm), loose patch clamp conditions exist where the neuronal dendrites represent the patch of cell membrane

Translation The process in which the genetic information from RNA is translated into specific amino acid chain, protein or polypeptide before further editing and/or folding for the final protein product to perform specific function within the cell, tissue or organism

Truncation A mutation which induces premature stop codon thereby producing a shorted protein with a truncated (aborted) tail

Type I pheromone A major group of moth sex pheromones composed of a 12–18 carbons-long fatty acid chain (with one, two or three double bounds and trans (*E*) or cis (*Z*) isomers) connected to an oxygenated functional group (acetate, alcohol

or aldehyde) as the only polar and therefore hydrophilic (water loving) portion of the molecule

UDP-Glycosyltransferase An enzyme that catalyzes the addition of a glycosyl group from a uracyl-diphosphate (UDP) sugar molecule to a small hydrophobic (water hating) fatty acid chain

Varroa destructor An external parasitic mite that can only lives attached to the body of honey bees, spreading varroosis disease and deformed wing virus in the colony or hive

Vector An agent or organism (invertebrate arthropod such as insect) that carries and transmits an infectious pathogen reponsible for epidemic disease into another living organism such as human

Visual pigment (rhodopsin) A G-protein coupled receptor molecule consisting of a protein (opsin) and a vitamin A-derived chromophore (11-cis retinal) that plays a key role in image formation in visual receptor neurons in both *Drosophila* and human eyes

Volatile organic compound (VOC) An organic chemical that has a high vapor pressure and low boiling point at normal temperature, which causes the chemical molecule to easily change to gas from the liquid or solid site of production and evaporate into the surrounding air (volatility); it probably includes most naturally-occurring odorants, most scents, odors or perfumes that play a key role in communication between plants and between plants and other organisms (including insects); for instance, specific subset of VOCs or green leaf volatiles that are released by damaged plants upon herbivore attacks in order to attract the herbivore natural enemy (predator) while alerting the other plants about the herbivore attacks

Voltage clamp An electrophysiological method for measuring the current across a cell membrane at a fixed voltage across the membrane

Wolbachia The most common inherited parasitic endosymbiotic bacterial species naturally present in more than 60% of insect species (including wasps and mosquitoes); the *Wolbachia*-mediated infection can result in cytoplasmic incompatibility and embryonic mortality in specific insect pest species

Xenobiotic A drug chemical substance that is foreign (exterior) to a biological system

Xenobiotic metabolizing enzyme A family of enzymes that modulate cellular interaction with environmental xenobiotic chemicals (insecticides or toxic pollulants) by degradation or modification (recycling) of the xenobiotic chemical structure

Zeitgeber An external environmental factor (e.g length of daylight or temperature) that helps setting (or re-setting) the rythm of a biological clock